SPECIFIC IMMUNOTHERAPY

FOR

RENAL CELL CARCINOMA

Ivar Bleumer

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FOR

RENAL CELL CARCINOMA

Een wetenschappelijke proeve op het gebied van de Medische Wetenschappen

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CONTENTS

LIST OF ABBREVIATIONS

5-FU	5-fluorouracil
ADCC	antibody dependent cellular cytotoxicity
allo-SCT	allogeneic stem cell transplantation
ANC	absolute neutrophil count
APC	antigen presenting cell
BAY	BAY-43-9006
CA9	carbonic anhydrase-IX$^{MN/G250}$
CA9p254	HLA-A2.01-restriction (nonamer)
CA9p249	HLA-DR-restriction (20-mer)
CD	cluster of differentiation
ccRCC	clear-cell RCC
CNS	central nerve system
CR	complete response
CRA	cis-retinoic acid
CT	computerized tomography
CTL	cytotoxic T-lymphocyte
DC	dendritic cell
DIL	DTH infiltrating lymphocytes
DLI	donor lymphocyte infusion
DTH	delayed-type hypersensitivity
EORTC	european organization for research and treatment of cancer
FDA	food and drug administration (USA)
GLUT	glucose transporters
GM-CSF	granulocyte-macrophage colony stimulating factor
GMP	good manufacturing practice
GVHD	graft versus host disease
HACA	human antibody chimeric antibody
HIF	hypoxia inducable factor
HIV	human immuno-deficiency virus
HLA	human leucocyte antigen
HPLC	high performance liquid chromatography
i.v.	intravenously
iDC	immature dendritic cells

IFN	interferon
IL	interleukin
LAK	lymphokine activated killer
LD-IL-2	low-dose interleukin-2 pulsing schedule
LN	lymph node
LU	lytic units
mAb	monoclonal antibody
mDC	mature dendritic cells
MDR1	multidrug resistance
MHC	major histocompatibility complex
MIU	million units
MPA	medroxyprogesterone acetate
MRC	medical research council collaborators
mRCC	metastasized RCC
MRP	multidrug resistance-associated protein
NK	natural killer cell
PBMC	peripheral blood mononucleart cells
PBL	peripheral blood lymphocytes
PBS	phosphate buffered saline
Pca	prostate cancer
PD	progressive disease
PGE	prostaglandin
P-gp	p-glycoprotein
PR	partial response
PSA	prostate specific marker
pVHL	protein VHL
RCC	renal cell carcinoma
RIT	radio-immunotherapy
s.c.	subcutaneously
SAE	serious adverse event
SD	stable disease
TAA	tumor associated antigen
TGF	tumor growth factor
TIL	tumor infiltrating lymphocyte
TNF	tumor necrosis factor

TNM	tumor-node-metastasis
ULN	upper limit of normal
VEGF	vascular endothelial growth factor
VHL	von Hippel-Lindau
VLB	vinblastin
WMSL	weighted mean of specific cytolysis
WX-G250	chimeric monoclonal antibody G250

THIS THESIS IS BASED ON THE FOLLOWING ARTICLES:

Immunotherapy for renal cell carcinoma.
Eur Urol. 2003 Jul;44(1):65-75.

Tumor antigens and markers in renal cell carcinoma.
Urol Clin North Am. 2003 Aug;30(3):455-65.

Validation of an integrated staging system toward improved prognostication of patients with localized renal cell carcinoma in an international population.
J Urol. 2003 Dec;170(6 Pt 1):2221-4.

Vaccination of patients with metastatic renal cell carcinoma with autologous dendritic cells pulsed with autologous tumor antigens in combination with interleukin-2: a phase 1 study.
J immunother. 2002 nov-dec;25(6):500-8.

Vaccination of patients with progressive renal cell carcinoma with CA9 peptide pulsed mature dendritic cells.
Accepted for publication in the Journal of Immunotherapy.

A phase II trial of chimeric monoclonal antibody G250 for advanced renal cell carcinoma patients.
Br J Cancer. 2004 Mar 8;90(5):985-90.

A clinical trial with chimeric monoclonal antibody WX-G250 and low-dose interleukin-2 pulsing scheme for advanced renal cell carcinoma patients.
J Urol. 2006 Jan; 175:57-62

CHAPTER 1

GENERAL INTRODUCTION
&
SCOPE OF THIS THESIS

The General Introduction is based on the follwing articles

Immunotherapy for renal cell carcinoma.
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1.1 Epidemiology & Etiology of RCC

Renal cell carcinoma (RCC) is the most common renal tumor, the third malignancy within urological oncology and comprises 2-3% of all malignancies. RCC was conventionally thought to arise primarily from the proximal convoluted tubules. Indeed, this is the case for most of the clear-cell subtype of RCC (ccRCC), which accounts for 70% to 80% of all RCCs . However, other histological subtypes e.g. papillary or chromophobic RCC, typically arise from more distal components of the nephron. RCC occurs twice as often in men compared to women and the highest incidence of RCC is seen in the 6th decade [1,2]. The incidence of RCC is rising. Review of over 10.000 cases of RCC gathered in the Connecticut Tumor Registry indicate a six-fold increase in the incidence of RCC from 1935-1989; both in woman and men [3]. Several risk factors have been described for RCC. Tobacco smoking doubles the risk of RCC and there is a positive linear relation between body weight and the risk for RCC, especially in women. Other factors associated with higher risk for RCC are exposure to asbestos or chemicals, thiazide drug intake and urinary tract infections [4].
In the onset of RCC there are only few early warning signs. The classical triad of Virchow, consisting of an abdominal mass together with flank pain and macroscopic hematuria, is nowadays only seen in approximately 5% of the new cases presenting with RCC. If any, the presentation of the disease is accompanied with non-specific signs as fatigue, weight loss, malaise, fever and/or night sweats [5]. At present, more then 50% of all RCC are found incidentally. The incidence of these so-called incidentalomas has risen from 15 to over 50% in the past decades. Typically, these incidentalomas are of smaller size, often confined within the renal capsule and associated with a favorable clinical outcome.

1.2 Prognostic factors and staging systems

In order to adequately assess the success rate of current and future treatment modalities, continuous evaluation of prognostic factors and staging systems is essential. Historically, several staging systems have been used in parallel. The realization that one international staging system for RCC is important has led to the general acceptance of the current tumor-node-metastasis staging system (TNM) published in 2002 [6] (table 1).

Table 1
TNM staging system 2002 [6]

T1b	Tumor greater than 4.0 cm, but no more than 7.0 cm, in greatest dimension, limited to the kidney.
T2	Tumor greater than 7.0 cm in greatest dimension, limited to the kidney.
T3	Tumor extends into major veins, or invades adrenal or perinephric tissues but not beyond Gerota's fascia
T3a	Tumor invades adrenal gland or perinephric tissues but not beyond Gerota's fascia.
T3b	Tumor grossly extends into renal vein(s) or vena cava below the diaphragm.
T3c	Tumor grossly extends into vena cava above the diaphragm.
T4	Tumor invades beyond Gerota's fascia.
N0	No regional lymph node metastasis.
N1	Metastasis to a single regional lymph node.
N2	Metastasis in more than one regional lymph node.
M0	No distant metastasis.
M1	Distant metastasis.

The TNM staging system addresses the anatomic extend of the tumor and recognizes 4 subdivisions. I: T1N0M0, II: T2N0M0, III: T3N0-1M0 and T1-2N1M0, IV: T4N0-2M0-1 and any TN2M0 and anyTN0-1M1 [7] (table 2).

At present, tumor stage is still the most important prognostic factor for the clinical outcome of RCC [8]. Nevertheless, the natural history of RCC is complicated and influenced by numerous factors. Accurate prediction of clinical outcomes of patients with RCC immediately after resection of localized disease would be beneficial for counseling, follow-up evaluation and identification of high risk patients potentially suitable for adjuvant therapies and clinical trials. It has become increasingly clear that the clinical behavior of RCC results from interactions among multiple variables. As a result, nomograms and integrated staging systems have been developed to improve the predictive capabilities of physicians. Several of these systems, in particular the UCLA integrated staging system, are discussed in chapter 2 of this thesis.

Table 2
Subdivisions of the TNM staging system and 5-year survival [7]

Stage	*T*	*N*	*M*	*5-year survival*
I	1	0	0	91%
II	2	0	0	74%
	1	1	0	
III	2	1	0	67%
	3	0, 1	0	
	4	0, 1	0	
IV	Any	2	0	32%
	Any	Any	1	

1.3 Tumor markers

A new development in cancer research is the identification of molecular tumor markers. Tumor markers can be of great value in several aspects of cancer treatment. For prostate cancer (PCa) the use of markers has become daily routine.

Prostate specific antigen (PSA) has long since been used as a serum tumor marker to detect

relapse of PCa [9]. Moreover, PSA is used for population screening and diagnosis. Recently, DD3^{PCA3} has been described as the most PCa-specific gene that is strongly over-expressed in >95% of primary PCa specimens and in PCa metastasis [10,11]. Evaluation of a time-resolved fluorescence-based quantitative RT-PCR assay for the detection of DD3^{PCA3} transcripts in urinary sediments obtained after extensive prostatic massage showed a high negative predictive value for the presence of PCa [12]. The use of this PCa-specific marker may have a great impact for the reduction of the number of prostate-biopsies currently needed to detect PCa.

Also for RCC numerous studies have been published that describe the potential use of tumor-associated biomarkers in the diagnosis and prognosis of RCC and as targets for (gene-immuno) therapy strategies. Tumor markers can be classified according to their origin and function.

- Apoptotic markers
- Proliferation markers
- Cell adhesion markers
- Tumor associated antigens
- Angiogenisis
- Cytogenetics

1.3.1. Apoptotic regulators & cell cycle proteins

As mentioned, RCC shows a high degree of resistance to chemotherapy and radiation [13,14]. The loss of control of apoptosis may contribute to progression and resistance to treatment modalities and can be attributed to an interaction between p53 and the apoptotic regulators bcl-2 and Bax [15]. However, the majority of RCC express normal function of the protein p53 and analysis of expression of p53, bcl-2, or Bax could not be significantly correlated with other parameters examined including tumor recurrence, metastasis, or survival rate [16].

Aberrations in the G1-S transition have been observed in a variety of tumors, suggesting that cell cycle defects are related to the activation of oncogenes and inactivation of suppressor genes involved in the transformation process. The frequency of G1/S aberrations in RCC has not been fully clarified. Cyclins (A and D1), pRb, p21 (waf1/cip1) and p27 (KIP1) are cellular proteins involved in the tight regulation of cell cycle events. Several groups have studied the relation between these markers and clinical and histopathological parameters as well as to clinical outcome [17-21]. Review of these studies suggests that Cyclin-A, but mainly low expression p27 is associated with poor prognosis for patients with RCC.

1.3.2. Proliferation Markers

Proliferation of cells is unmistakably related to cancer and subsequently, several proliferation markers have been evaluated. AgNOR (Silver stained nucleolar-organizing regions) reflects transcription activity of ribosomal DNA and mitotic activity. PCNA (Proliferating cell nuclear antigen) is a protein synthesized in the later G1 and S phases of the cell cycle. Finally, Ki-67 (MIB1) is a monoclonal antibody that stains a proliferating-specific antigen in tumor cells. All these markers have been correlated to histological grade and stage and even survival [22-24]. Comparative studies that have been performed suggest that Ki-67 is the more powerful prognostic factor of the three [25,26]; nevertheless more multivariate studies are needed.

1.3.3. Cell Adhesion Markers

Cancer metastasis is a complex multistage process. Decreased intercellular adhesion enables detachment of tumor cells and can play a role in the early steps of the metastatic process. Cell adhesion can be mediated through at least four families of adhesion molecules (intergrin, immunoglobulin, selectin, and cadherin). Also the tubular epithelium, from which the RCC originates, expresses a complex set of adhesion molecules. During carcinogenisis, the combination of cadherin expression changes in almost 50% of RCC [27]. E-cadherin, a Ca++-dependent epithelial cadherin, is considered as a critical molecule for epithelial integrity [28]. However, most RCCs do not express E-cadherin because renal proximal tubular epithelium from which RCCs originate does not express E-cadherin. In contrast, other studies showed that in normal kidney tubular epithelium N-cadherin and cadherin-6 are expressed [29,30] and that expression patterns may be related to the metastatic behavior of the tumor. The cadherin function is modulated through cytoplasmic proteins termed catenins. Immuno-histochemical staining revealed that catenins were expressed in all the segments of the nephron including proximal tubules. The catenin family seems to be less divergent than the cadherin family. Therefore, it was reasoned that there might be a correlation between aggressiveness of RCC and a decreased expression of alpha-catenin, which is a member of the catenins that link cadherin to the cytoskeleton. Immuno-histochemical staining on RCC using antibodies against E-cadherin and alpha-catenin has revealed that the ratios of abnormal staining for E-cadherin and alpha-catenin were 77% and 37%, respectively. The prognostic value of E-cadherin is controversial. However, a significant correlation between survival and decreased expression of alpha-catenin was observed. Whether alpha-catenin immuno-histochemistry provides additional prognostic information remains to be established.

Although cell adhesion molecules are usually membrane-bound, soluble forms (sICAM-1, sVCAM-1 and sELAM-1) exist and are generated by shedding of the extracellular portions

Table 3
Overview of tumor markers in renal cell carcinoma

Renal Cell Carcinoma	Tumor markers	Reference
Apoptotic regulators	p53 bcl-2 Bax	15,16,22
Cell Cylcle proteins	cyclin A and D1 p21 (waf1/cip-1) p27 (KIP1) pRb	17-21
Proliferation markers	AgNOR PCNA Ki-67 (MIB1)	22-26
Cell adhesion markers	E-cadherin N-Cadherin Cadherin-6 α-catenin sICAM1 sVCam-1 sECam-1 CD44	27-30 31,32 33,34
Tumor associated antigens	RAGE CA-IX$^{G250/MN}$	50 52,57
Angiogenic markers	Intratumoral microvascular density VEGF	35,36 37,38
Cytogeic markers	Karyometry Myc	39-42 44,46

from the cell surface. These molecules have been investigated along with other clinical parameters in patients with metastatic RCC [31,32]. The prognostic significance of sICAM-1 might indicate a role of this molecule for tumor progression, potentially in association with the abrogation of anti-tumor immune responses. The possibility of defining a pretreatment risk model based on sICAM-1 level, ESR and CRP also warrants further investigation, with regard to a possible linkage between acute phase proteins and sICAM-1 levels.

CD44 is a transmembrane glycoprotein involved in cell-cell and cell-matrix interactions. De novo expression of CD44 and its variant isoforms have been associated with aggressive behavior in various tumors [33,34]. Since few data are available concerning the role of CD44 in the biological behavior of locally confined renal tumors, the expression of CD44 in a large set of

conventional RCC was analyzed to determine its prognostic value in association with other clinico-pathologic variables [34]. The investigators concluded that CD44 expression was correlated with progression and survival of RCC patients and can be considered as a useful prognostic parameter in conventional renal cell carcinoma and may be used in evaluation of the outcome of these tumors.

1.3.4. Angiogenic Markers

Neovascularization is inextricable related to tumor growth and metastatic dissemination. Nevertheless, angiogenisis measured as intratumoral microvascular density is not an independent prognostic factor in RCC [35,36].Vascular endothelial growth factor (VEGF) is definitely related to histological grade and stage, the prognostic value remains uncertain [37]. Besides its association with grade and stage, VEGF may also play a role in the treatment of RCC. A recent publication described the effect of bevacizumab, a neutralizing antibody against vascular endothelial growth factor (VEGF), in the treatment of mRCC [38]. In this randomized, double blind phase 2 trial, placebo was compared with 2 doses of bevacizumab. The primary endpoint of this study was the time to progression of disease. According to intention-to-treat analysis, progression-free survival in the group receiving the high dose bevacizumab (10mg/kg) was significantly longer (median: 4.8 months) than in the placebo group (median: 2.5 months, $P<0.001$ by the log-rank test). The NCI data safety and monitoring board recommended closure of accrual on the basis of these differences in time to progression, despite an overall response rate of approximately 10%, and no difference in survival between the three study-arms.

1.3.5. Cytogenetic Markers

Recently it has become widely accepted that genetic alterations play an important role in the development of many cancers. The relationship of abnormal nuclear morphology to molecular genetic alterations in RCC is unknown. Nuclear morphometric analyses have been used successfully to predict the outcome of patients with cancer when classical pathologic grading systems failed. Indeed, several investigations showed a significant correlation between morphometric parameters and survival of patients with RCC [39-42]. Nevertheless, in colorectal carcinoma, it was shown that nuclear morphology seemed not to be directly influenced by the individual genetic alterations but was by fractional allelic loss (i.e., a global measure of genetic changes) [43]. Thus, it might be suggested that complex tumor properties such as pathologic appearance and metastatic potential cannot be understood unless most of the underlying genetic factors are taken into consideration. Therefore several chromosomal abnormalities and subsequent over-expression of proteins have been evaluated. L-myc and C-myc, for instance,

are a family of proto-oncogenes that have been studied in RCC [44]. yet, so far only associations could be made with histological grade and stage, without showing independent prognostic significance [44-46].

1.3.6. Tumor-Associated Antigens: CA-IX$^{G250/MN}$

Markers that are in particular of interest for in the optimalization of gene-immunotherapy are RCC-associated antigens. However, despite compelling evidence that RCC is an immunogenic tumor, until recently only a few specific tumor antigens, such as RAGE, are known. RAGE, initially defined through CTL technology, is expressed in a minor percentage of RCC, and therefore a sub optimal target [47-51]. In this aspect the identification of the RCC-associated antigen CA-IX$^{G250/MN}$ (CA9) is of interest.

CA9 is expressed in >95% of all ccRCC tumors. Moreover, no expression can be detected in normal kidney tissue, including fetal kidney, and in other normal tissues the expression is highly restricted and limited to large bile ducts and gastric epithelium [52]. Furthermore, CA9 is also expressed in several non-RCC tumors. In RCC, the chimeric monoclonal antibody G250 (WX-G250) which recognizes the CA9 antigen, has been identified and developed for both therapeutical and diagnostical purposes [52]. Furthermore, a CA9-derived peptide recognized by HLA-A2.1 restricted CTL and a helper peptide recognized by HLA-DR restricted T-helper cells have been identified [53,54]. These findings, together with the high prevalence of CA9 in RCC make this antigen a promising tool for specific immunotherapy strategies for RCC [55,56].

The high expression of CA9 in ccRCC implies the involvement of CA9 in renal carcinogenisis. CA9 is a member of the carbonic anhydrase family, which are enzymes involved in acid-base balance, CO2 transport and ion exchange, which explains their physiological expression in the gastric mucosa. Still, the involvement of CA9 in the carcinogenis remains to be elucidated. CA9 cDNA transfection into CA9 negative cell lines did not alter the cell doubling time and did not lead to immortilazation (pers. communication). Thus, CA9 seems not to function as an oncogene. It is known that the acidic environment around tumors favor their malignant behavior, and this might explain the expression of CA9 in a variety of tumors. However, it is also conceivable that CA9 is up regulated only to correct the carcinogenic acidic environment, instead of creating it.

Using the clinical and data resources of the UCLA Kidney Cancer Program, Bui et al investigated whether CA9 is associated with progression and survival [57]. Immunohistochemical analysis using a CA9 mAb was performed on tissue microarrays from patients treated by nephrectomy for ccRCC. CA9 staining was correlated with response to treatment, clinical factors,

pathological features, and survival. Low CA9 staining was an independent poor prognostic factor for survival for patients with metastatic RCC, with a hazard ratio of 3.10 ($P < 0.001$). CA9 significantly substratified patients with metastatic disease when analyzed by T stage, Fuhrman grade, nodal involvement, and performance status ($P < 0.001$, $P = 0.001$, $P = 0.009$, and $P = 0.005$, respectively). Overall expression of CA9 decreased with development of metastasis, as demonstrated by the lower CA9 staining levels in metastatic lesions relative to matched primary tumor specimens ($P = 0.036$). On the basis of these data, CA9 seems to be the most significant molecular marker described in kidney cancer, to date.

1.4 The von Hippel-Lindau Gene and RCC

Von Hippel-Lindau disease is a hereditary multisystem cancer syndrome caused by germline mutations of the VHL tumor-suppressor gene on the short arm of chromosome 3 (reviewed in [58]. The disease predisposes to the development of a variety of highly vascularized benign and malignant tumors of the central nervous system, adrenal glands, pancreas reproductive adnexal organs and the kidney. VHL-disease is the principal cause of inherited RCC. Furthermore, in almost 100% of the sporadic ccRCC there is an inactivation of the VHL-gene either by mutation, deletion or methylation [59,60].

VHL plays a central role in the cellular oxygen homeotasis. In normoxic conditions the VHL-protein (pVHL) binds and deactivates the hypoxia-inducible factor-1a (HIF-1a). However, in hypoxic condition pVHL does not target HIF-1a for degradation leading to an increased transcription of a variety of HIF-regulated genes. Tumors, e.g. ccRCC, in which both alleles of the VHL gene are inactive mimic the hypoxic situation; leading to an overproduction of HIF-1a and subsequently the HIF-1a targeted genes (VEGF, GLUT1, TGF-a, CA9), several of which have been discussed in this chapter [61,62]. Besides cacinogenisis through the HIF-1a pathway, the VHL-status has also been associated with effects on apoptosis (Bcl-pathway) and cell cycle events (p27, cyclin-D). Many of these HIF-targeted genes are associated with aggressive tumor growth, suggesting that gene products of the VHL/HIF-1a pathway are important prognostic factors. Furthermore, the same VHL/HIF-1a pathway is of interest to develop molecular therapeutic interventions (discussed in [63]).

1.5 Summary of Tumor Markers

Tumor markers are mainly used to diagnose specific malignancies. The methods commonly involve immuno-histochemistry and cytogenetics, including fluorescent in situ hybridisation (FISH), and reversed transcriptase (RT) and polymerase chain reaction (PCR). In RCC several investigated tumor markers are promising (summarized in table 3). They may show additional prognostic value over classical prognostic factors like stage and grade. Also, these markers can finally be used for a better patient selection, development of specific gene-immunotherapy strategies and a better follow-up. Ultimately these factors should show its value in a prospective well-controlled manner and additionally, more research is needed to obtain new (better) antigens and markers in RCC. Also, the association with the carcinogenisis of RCC and the inactivation of the VHL-gene with subsequent over-expression of several tumor-associated genes is intriguing and should be elucidated.

1.6. Therapeutic approaches in RCC

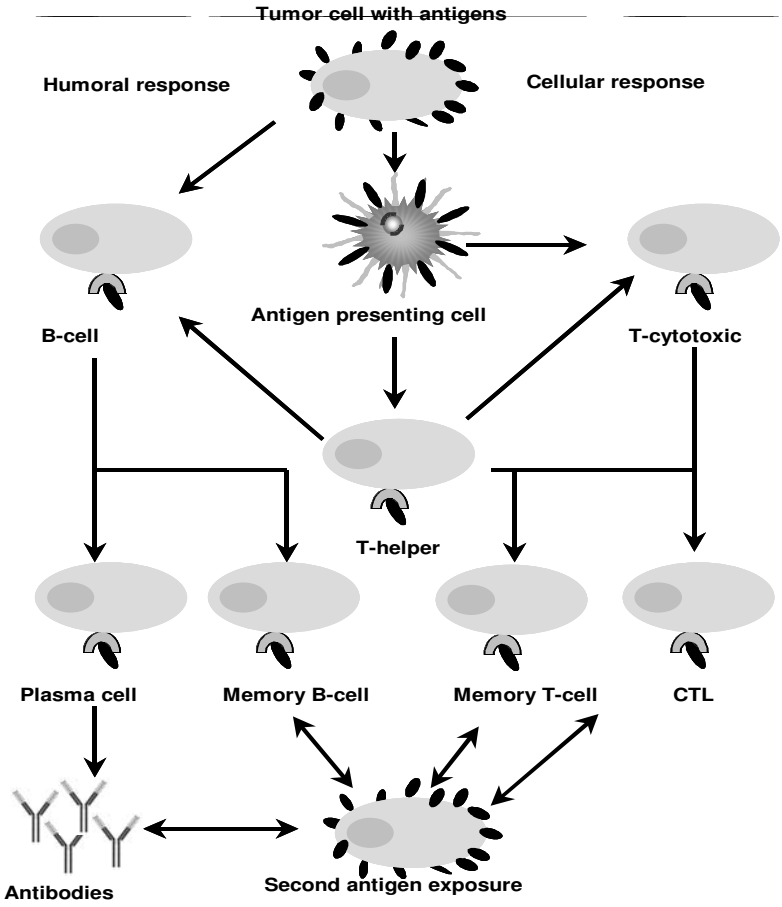
The treatment of RCC is dependent on the anatomic extend of the disease, as addressed by the TNM staging system. Localized disease, that is, if no metastatic disease is observed, is treated by means of surgical removal of the tumor. Nevertheless, up to 30% of all patients treated with curative intent will develop distant metastasis, especially patients within TNM stage III or IV. Review of 3502 patients with metastatic RCC (mRCC) treated with one of 72 chemotherapeutic agents revealed a cumulative objective response rate of only 2 - 6 % [64]. This is the consequence of intrinsic or acquired resistance of renal cancer cells to chemotherapeutic drugs, as discussed earlier. Combination therapy and/or radiotherapy does not significantly change response rates or survival [65]. Therefore, chemo- and radiotherapy are considered to be of limited value for the treatment of mRCC.
RCC is one of the few tumors where spontaneous regression of metastatic disease after tumornephrectomy has been documented [66]. Therefore, much attention has been focused on immunotherapeutical modalities for the treatment of mRCC.

1.7. The immune system

Already at the beginning of the twentieth century Paul Ehrlich suggested that cells are often undergoing malignant transformation ('aberrant germs') and that this process was controlled by the immune system. Later, Burnet and Thomas have further developed this concept as the immune surveillance hypothesis, speculating that the immune system was constantly searching for aberrant cells and consequently destroying them at pre-clinical stage. Furthermore, it was proposed that immune surveillance played a role in growth delay of established tumors. Recently, this concept has been refined and renamed immunoediting[67].

Current literature indicates that T-cells are probably the major factor for both cellular and humoral immunological control of tumor growth (figure 1). To activate the cellular immune pathway, class I and class II major histocompatibility complex (MHC) molecules bind to short peptides of 8-10 and 13-20 amino acids, respectively. This peptide-MHC complex is recognized by the T-cell receptor on the surface of T lymphocytes leading to an immunological synapse; whether a tumor-associated antigen (TAA)-derived peptide elicits a cellular T-cell response is regulated by the quality of this synapse.

Fig. 1
Overview of the humoral and cellular pathway of the immune system.



Humoral antitumor responses are regulated by antibodies that mediate their effect mainly through antibody dependent cellular cytotoxicity (ADCC). Tumor cell lysis by ADCC is regulated by the interaction between the Fc-region of an antibody bound to a tumor cell and the Fcg receptors on immune effector cells, such as neutrophils, macrophages and natural killer (NK) cells [68]. Interactions of NK cells with IgG antibodies via the FcgRIII receptor are well known to induce a signal transduction cascade leading

to ADCC as well as the release of various cytokines involved in anti-tumor responses [69]. The different immunotherapeutical modalities currently used and investigated to elicit immune responses against RCC will be reviewed.

1.8. Non-specific immunotherapy

1.8.1. Interferon

Interferons (IFNs) are cytokines that are already present under normal physiological circumstances albeit at low levels. Their expression is greatly enhanced after exposure to different stimuli, e.g. viruses. Three different classes of IFNs have been described: IFN-alpha, IFN-beta and IFN-gamma. The mechanisms of action of IFNs in clinical oncology have not been clarified, but some are thought to be stimulation of phagocytosis by macrophages, upregulation of MHC class I molecules and direct antitumor activities [70].

The clinical relevance of IFN-beta and IFN-gamma in the treatment of advanced RCC has not been proven [65,71,72]. For IFN-alpha, two large randomized studies of interest have been published. In the trial performed by the Medical Research Council Collaborators (MRC) [73], IFN-alpha (10 MIU three times weekly) was compared with 300mg of the orally administered hormonal drug medroxyprogesterone acetate (MPA). Treatment with IFN-alpha led to an improved 1-year survival of 12% (MPA 31% survival, IFN-alpha 43%), and an improvement in median survival of 2,5 months (MPA 6 months, IFN-alpha 8,5 months). A trial performed by Pyrhonen et al showed that the combination of IFN-alpha and vinblastine (VLB) was superior to VLB alone[74]. Since VLB has not shown to enhance the effect of IFN-alpha [75,76], the results of the latter trial must be due to the IFN-alpha treatment.

13-Cis-retinoic acid (CRA), a vitamin A derivative, can markedly upregulate the differentiation and proliferation of normal and neoplastic cells and synergistic effects of IFN and CRA have been described [77]. Motzer et al presented the results of a phase III trial comparing IFN-alpha alone with IFN-alpha in combination with CRA [78]. Patients received either daily subcutaneous (s.c.) injections of an escalation dose of IFN-alpha (3 MIU to 9 MIU), or this regimen combined with 1mg/kg CRA per day. Despite the higher response rate for the combination therapy (6% versus 12%) no difference was found in median survival time (15 months for all patients). The trend that sustained remissions for IFN-sensitive tumors were observed when combination therapy was given, needs to be confirmed with long-term follow-up data. A similar study (EORTC 30951) has been completed in January 2001 and the results will be mature within the second half of 2003.

1.8.2. Interleukin-2

Interleukin (IL)-2 is mainly produced by CD4+ T-cells, but also by CD8+ T-cells and large granular lymphocytes. The actions of IL-2 comprise the stimulation of e.g. NK-cells to secrete different cytokines like IFN-alpha, tumor necrosis factor (TNF-alpha) and granulocyte-macrophage colony-stimulating factor (GM-CSF), which activates the monocyte/macrophage lineage [79]. Consequently, differentiation of lymphokine-activated killer (LAK) cells [80], maturation of antigen presenting cells (APC) and enhanced ADCC [81] are induced. In 1995, Fyfe et al published the results obtained in 255 patients with mRCC treated with high-dose intravenous (IV) bolus regimens using doses of either 600.000 or 720.000 IU/kg. In this patient population, 15% showed an objective response (CR: 7%; PR: 8%). These results led to the USA Food and Drug Administration (FDA)-approved use of IL-2 in the treatment of mRCC [82]. Severe side effects like hypotension, pulmonary edema and renal dysfunction were observed during high-dose bolus IL-2 treatment with a treatment-related mortality of up to 4%. With the intention to diminish the severe side effects [83], clinical trials evaluating continuous high-dose intravenous infusion or subcutaneous (SC) administration of IL-2 were performed. Collective data showed a clear decrease in toxicity with comparable response rates [84,85]. However, no randomized studies of sufficient size have been published today.

Table 4
Overview of cytokine-based immunotherapy trials for renal cel carcinoma

Author [reference]	*# Pts*	*Treatment*	*Overall Response in %*	*Median Survival (months)*	*Survival Benefit*
MRC & Collab. [73]	335	IFN versus MPA	13 (IFN) 7 (MPA)	8.5 mo (IFN) 6.0 mo (MPA)	Yes
Pyrhonen [74]	160	IFN + V versus V	16.5 (IFN + V) 2.5 (V)	67.6 weeks (IFN + V) 37.8 weeks (V)	Yes
Motzer [78]	284	IFN + CRA versus IFN	12 (IFN + CRA) 6 (IFN)	15 mo (IFN + CRA) 15 mo (IFN)	No
Fyfe [82]	255	IL-2	14	16.3 mo	N/a
Negrier [88]	425	IL-2 versus IFN versus IL-2 + IFN	6.5 (IL-2) 7.5 (IFN) 18.6 (IL-2 + IFN)	13 mo (IL-2), 12 mo (IFN), 17 mo (IL-2 + IFN)	No
Atzpodien [91]	78	IL-2 + IFN + 5-FU versus T	39.1 (IL-2 + IFN + 5-FU) 0 (T)	24 mo (IL-2 + IFN + 5-FU) 13 mo (T)	Yes
Van Herpen [92]	52	IL-2 + IFN + 5-FU	12	16.5 mo	N/a
Negrier [93]	131	IL-2 + IFN + 5-FU versus IL-2 + IFN	8.2 (IL-2 + IFN + 5-FU) 1.4 (IL-2 + IFN)	12 mo (IL-2 + IFN + 5-FU) 12 mo (IL-2 + IFN)	No

Abbreviations: #Pts: number of patients, MRC: Medical Research Counsil, IFN: interferon-alpha, MPA: medroxyprogeterone acetate, mo: months, V: vinblastine, CRA: 13-cis-retinoic acid, IL-2: Interleukin-2, N/a: not applicable (no control study arm), 5-FU: 5-fluorouracil, T: tamoxifen.

1.8.3. Combination therapy

Different combinations of cytokines, e.g. IL-2 with IFN-beta [86], IFN-gamma and GM-CSF [87] have been studied in the treatment of mRCC. However, none as extensive as the combination of IL-2 and IFN-alpha. One of the most informative studies was conducted by the Groupe Francais d'Immunotherapie [88]. This is the only clinical trial so far in which patients were randomized between IL-2, IFN-alpha or the combination of the two cytokines. Patients received either (a) rIL-2, which was administered as a five-day continuous IV infusion at a dose of 18 MIU m-2 per day. The treatment schedule consisted of two induction cycles and four maintenance cycles, with a three-week rest period between cycles. Each induction cycle consisted of two five-day courses of IL-2 infusion separated by a six-day break. Each maintenance cycle consisted of a five-day infusion followed by three weeks of no therapy (b) 18 MIU of IFN-alpha 3 times a week for the 10 weeks SC, or (c) rIL-2 as described plus a reduced dose of IFN-alpha of 6 MIU 3 times a week. Higher response rates for the combination therapy were found (6.5%, 7.5% and 18.6% respectively) and at one year the event-free survival rates were 15, 12 and 20% respectively. Still, the overall survival did not differ significantly between the study arms.

Although treatment of mRCC with chemotherapy has no impact on survival [1], pre-clinical data have shown synergistic effects of 5-fluorouracil (5-FU), IFN-alpha, and IL-2 [89,90], which led to the initiation of several clinical trials evaluating this combination therapy. Atzpodien et al [91] performed a prospective randomized trial comparing the combination of IL-2, IFN-alpha and 5-FU (triple therapy) with hormone therapy (tamoxifen 80mg twice daily over 8 weeks) in 78 patients with mRCC. The treatment group showed an objective response rate of 39.1% (95%CI, 24.2-55.5) against no objective remissions in the group of patients receiving tamoxifen. However, several other trials have not been able to confirm the high response percentages of the triple therapy for mRCC (table 4) [92-94]. Currently, a definitive MRC/EORTC (30012) trial is ongoing comparing this regimen with IFN-alpha monotherapy.

Table 5
Risk factors for a short survival in patients with mRCC [98]

Bad Performance status	WHO > 1
Loss of weight	>10% in last 6 months
Short disease to treatment interval	< 2 years
Localization of metastasis	Metastasis in liver, bone or brains
Number of metastatic lesions	> 1 metastatic site
Nephrectomy	No nephrectomy (possible)
Laboratory parameters	
Sedimentation rate	>70 mmHg/h
LDH	>280 U/l
Neutrophiles	$>6x10^9$/l
Hemoglobine	<6.3 mmol/l

1.8.4.Long-term efficacy of non-specific immunotherapy

In 2000, the long-term survival update of patients who received high-dose bolus IL-2 was published [95]. Although the median survival for all patients was not altered (16.3 months), the median response duration for all objective responders was 54 months, with a range of 3 to >131 months. The median duration of the complete responders was not even reached (>80 months, range: 3 to 131+ months). Furthermore, 10% to 20% of the total number of included patients were estimated to be alive 5 to 10 years following IL-2 treatment. Similar long-term survival data have been observed with either IL-2 alone [96] or IL-2 in combination with IFN-alpha both with and without CRA and/or 5-FU [97]. These data indicate that cytokine-based therapy has the capacity to induce durable responses in a subset of patients. An important challenge is to select those patients most likely to benefit from cytokine-based therapy, emphasizing the need for large randomized trials comparing different cytokine regimes for patients within a variety of defined risk groups. Endpoints of these trials should be both survival and quality of life.

Table 6
Comparison of the survival of prognostic groups of patients with mRCC treated with immunotherapy or chemotherapy [98]

Prognostic groups (total points)	*Median survival (days) after treatment with*		
	IL-2	INF-alpha	Chemotherapy
Good (0 or 1)	570	652	352
Intermediate (2)	320	315	202
Poor (3)	177	193	158

Prognostic groups are defined as the sum of the number of points of three prognostic factors [99,101,102]. These prognostic groups were: (a) PS=performance status according to the World Health Organization: PS of 0 (fully active; able to carry on all pre-disease activities without restriction) = 0 points and PS of 1 (restricted in physically strenuous activity but ambulatory and able to carry otut work of light or sedentary nature) = 1 point; disease to treatment interval: 0=>24 months and 1=<24 months; number of metastatic sites: 0=one localization and 1= more than one localization.

1.8.5.Prognostic factors indicating the outcome of non-specific immunotherapy

As stated, it is important to define prognostic factors able to predict clinical response following cytokine-based therapy [98]. Several factors related to short survival of mRCC are shown in table 5 [82,88,99-101]. Based on the number of risk factors patients can be divided into prognostic groups to compare clinical results of patients receiving immunotherapy. This has been done for patients who received IL-2 [101,102] and IFN-alpha [103], and particularly patients with a good performance status, only one metastatic lesion and an interval from diagnosis to systemic therapy of more than 24 months showed a greater survival (table 6).

The role of nephrectomy in the treatment of mRCC has long been debated. However, recently two trials clearly showed survival benefit for patients undergoing nephrectomy prior to cytokine treatment [104,105]. Because this leads to a therapeutic advantage in only a minority of patients undergoing immunotherapy, tumornephrectomy before treatment remains controversial. Bex et al [106] evaluated immunotherapy with the primary tumor in place and tumorneph-

rectomy was only performed when a clinical response or stabilization was observed. Although this study was underpowered, a substantial number of patients were spared surgery. In short, the fact that immunotherapy in combination with the removal of the primary tumor reveals a time to progression- and a survival benefit, has been well established. However, a randomized trial is needed to evaluate the precise timing of cytoreductive therapy in combination with immunotherapy with regard to morbidity, overall survival and quality of life.

1.9. Adoptive cellular therapy

1.9.1. Lymphokine activated killer cells & Tumor infiltrating lymphocytes.

An alternative method to induce immune responses against RCC is the transfer of immune effector cells with antitumor reactivity. Two of these so-called "adoptive cellular therapy" approaches are LAK and TIL [107]. LAK cells are lymphoid cells found in the population of the large granular lymphocytes of the peripheral blood, which can be activated following in-vitro stimulation with supraphysiological concentrations of IL-2 and can subsequently be reinfused in the patient. LAK cells mediate antitumor activity in a non-specific manner. TIL cells are a population of lymphocytes infiltrating the tumor tissue that contain both non-specific cells (e.g. NK-cells), but also tumor specific cytotoxic T cells (CTL). TIL cells can be isolated from the tumor specimen, expanded ex-vivo in IL-2, and reinfused into the patient. The above-mentioned therapies showed great potential in in-vitro settings and animal models and led to an improved understanding of tumor-immunology. Nevertheless, randomized clinical trials comparing these new treatment options with IL-2 and/or IFN-alpha showed no additional clinical benefit [107].

1.9.2. Allogeneic stem cell transplantation

A new and effective form of adoptive cellular therapy against various forms of hematological malignancies is allogeneic stem cell transplantation (allo-SCT). In the setting of allo-SCT, T-cell reactivity against minor histocompatibility antigens is the suggested mechanism of action [108]. This method has also been explored for mRCC. In two comparable studies safety, feasibility and clinical results of allo-SCT after non-myeloablative chemotherapy were evaluated [109,110]. Both trials show that a graft-versus-tumor effect can be induced with objective response rates of 53% and 33% respectively. However, substantial toxicity due to graft-versus-host disease (GVHD) occurred and a transplantation-related mortality of 33% and 12% was observed in the two trials respectively. T cell depleted bone marrow may facilitate graft take

while preventing GVHD and subsequent donor lymphocyte infusions (DLI) may give rise to an anti-tumor effect which does not necessarily parallel GVHD [111]. These observations have formed the basis of a clinical study ongoing at our institution that evaluates the progression-free survival of partially T cell depleted allo-SCT followed by DLI in patients with poor risk lymphoma, colorectal carcinoma and mRCC.

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Outline of the thesis

Despite the fact that evaluation of cytokine-based therapies for mRCC shows that a subset of patients react favorable to immunotherapy, significant side effects do occur. With the increased knowledge of tumor-immunology, the recognition of immunogenic tumor proteins and antibodies, new treatment options with increased specificity and subsequently less side-effects are of interest. In this thesis several studies have been performed to evaluate these new treatment options.

Chapter 2 discusses the need for a staging sytem that adequately discriminates patients with RCC into prognostic groups in order to provide improved counseling, follow-up evaluation and identification of high risk patients potentially suitable for adjuvant therapies and clinical trials. This chapter presents a clinical algorithm using 3 well-known prognostic factors that can be used to predict survival and stratify patients undergoing nephrectomy for localized disease into 3 risk groups. External data from 2 outside institutions suggest the validity of this algorithm with a high concordance index. These risk categories can be used in clinical trial design and interpretation as well as in clinical management areas such as surveillance.

Chapters 3 & 4 discuss dendritic cell-based immunotherapy. Dendritic cells (DC) are the most potent antigen presenting cells (APC's) of the immune system with the ability to stimulate naive resting T cells to proliferate and differentiate into activated cytotoxic T cells specific against the presented antigen. Two clinical trials were performed evaluating safety and clinical or immunological responses of DC-based vaccines.

Chapters 5 & 6 deal with monoclonal antibody-based immunotherapy. Currently, several monoclonal antibodies (mAb) are used in cancer treatment. In RCC the chimeric mAb G250 (WX-G250) has been identified and developed for both therapeutical and diagnostical purposes. Antibody dependent cellular cytotoxicity (ADCC) has been suggested to be the main effector mechanism of WX-G250 and in-vitro data showed that interleukin-2 (IL-2) has the capacity to up-regulate WX-G250 mediated ADCC. This led to the initiation of two successive clinical trials evaluating infusions of WX-G250 as monotherapy and the combination of WX-G250 with a low-dose IL-2 pulsing regimen

CHAPTER 2

VALIDATION OF AN INTEGRATED STAGING SYSTEM TOWARDS IMPROVED PROGNOSTICATION OF PATIENTS WITH LOCALIZED RENAL CELL CARCINOMA IN AN INTERNATIONAL POPULATION

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2.1 Abstract

Purpose: Outcome prediction for patients with renal cell carcinoma is based on a combination of factors. In this study, a previously published clinical outcome algorithm based on 1997 T stage, Fuhrman grade, and performance score is validated using an international database.

Methods: A total of 1060 patients from 3 databases (St Radboud = SR, MD Anderson= MDA, and UCLA) who had localized renal cell carcinoma were evaluated for outcome prediction using a clinical outcome algorithm previously shown to stratify patients into 3 risk groups (low, intermediate, and high). Validation was performed by comparing the 3 risk groups separately within the 3 centers as well as by comparing the hazard ratios and concordance indices amongst the 3 centers.

Results: Estimated disease-specific survival (DSS) rates at 5 years for the low-risk groups were 94% (SR), 92% (MDA), and 93% (UCLA). The 5-year DSS rates for the intermediate-risk groups were 65% (SR), 73% (MDA), and 78% (UCLA), while the rates for the high-risk groups were 40% (SR), 30% (MDA), and 48% (UCLA). The concordance indices for each of the databases were 79% (SR), 86% (MDA), and 84% (UCLA).

Conclusions: A clinical algorithm that uses only 3 prognostic variables (1997 T stage, Fuhrman grade, and performance status) to stratify patients with localized renal cell carcinoma into 3 risk groups has been shown to be applicable to external databases. This algorithm may be useful for patient counseling, surveillance, and identification of high-risk patients for enrollment into clinical trials.

2.2 Introduction

The natural history of renal cell carcinoma (RCC) is complicated and influenced by numerous factors [1]. Accurate prediction of the clinical outcomes of patients with RCC immediately after resection of localized disease would be beneficial for counseling, follow-up evaluation, and identification of high-risk patients potentially suitable for clinical trials. It has become increasingly clear that the clinical behavior of RCC results from interactions amongst multiple variables. As a result, nomograms and integrated staging systems have been developed to improve the predictive capabilities of physicians. Nomograms based on the Cox proportional hazards model predict survivorship patterns for individual patients. By comparison, staging systems divide the patient population into subgroups where patients in different subgroups are predicted to have different survival patterns.

In 2001, Memorial Sloan-Kettering introduced a postoperative nomogram which assigns points based on a combination of variables that include histology, tumor size, 1997 T stage, and whether the patient presented with symptoms [2]. In the same year, UCLA introduced the UCLA integrated staging system (UISS) which combined 1997 TNM stage (I-IV), Eastern Cooperative Oncology Group (ECOG) performance status (PS), and Fuhrman grade [3]. Subsequent to the introduction of the UISS, Zisman et al simplified the staging system into a clinical outcome algorithm based on 1997 T stage, ECOG PS, and Fuhrman grade; this UISS simplified algorithm resulted in 3 risk groups (low, intermediate, and high) for patients with RCC [4]. More recently, Mayo clinic introduced the SSIGN prediction model based on 1997 TNM stage, tumor size > 5 cm, nuclear grade, and tumor necrosis [5]. The SSIGN model divides patients into 10 different subgroups.

These predictive models may provide valuable information with regards to prognostic factors associated with survival. In this analysis, the UISS simplified outcome algorithm is applied to patients from University Hospital St Radboud (Netherlands -177 patients), MD Anderson (399 patients) and an updated UCLA database (484 patients) in an effort to determine whether such an algorithm can stratify patients from different institutions into distinct survival groups. A total of 1060 patients with clinically localized RCC who underwent nephrectomy were available for evaluation of the outcome algorithm.

2.3 Methods

A previously published outcome algorithm [3] was applied to databases from the St Radboud (Netherlands), MD Anderson (MDA), and UCLA (updated database) in an effort to stratify patients into distinct survival groups. Briefly, stage was determined according to the 1997 UICC TNM classification of renal tumors [6]. Tumors were classified according to the Heidelberg classification [7] and graded according to the Fuhrman grading scheme [8] by pathologists from each of the 3 institutions. Eastern Cooperative Oncology Group performance status (ECOG PS) was determined according to the original criteria set forth by Oken et al. [9]. A total of 1060 patients from 3 institutions underwent radical or partial nephrectomy. All patients with localized disease who had no evidence of nodal involvement or metastatic spread were included from each center. Risk groups were defined according to 1997 T stage, Fuhrman grade, and ECOG PS. A decision box was constructed (see appendix) to permit a simple algorithmic assignment of patients into low intermediate, and high-risk groups.

Each of the databases from the institutions was updated in a retrospective manner. For all 3 institutions, the ECOG PS for each patient was determined at the time of office visit before nephrectomy.

Disease-specific survival was determined by the Kaplan-Meier method and was calculated from the date of nephrectomy to date of death or last follow-up. Comparisons among groups were performed by the log rank method to determine the significance of the Kaplan-Meier curves.

Table 1
Characteristics of patients with localized disease

	St Radboud (N = 177)	*MD Anderson (N = 399)*	*UCLA (N = 484)*
Mean age	59.6	58.1	61.5
Males	121 (68%)	265 (66%)	309 (64%)
Fuhrman grade 1	38 (21%)	25 (6%)	101 (21%)
Fuhrman grade 2	86 (49%)	152 (38%)	267 (55%)
Fuhrman grade 3	43 (24%)	183 (46%)	100 (21%)
Fuhrman grade 4	10 (6%)	39 (10%)	16 (3%)
ECOG 0	165 (93%)	312 (78%)	282 (58%)
ECOG 1	12 (7%)	87 (22%)	197 (42%)
T1	58 (33%)	157 (39%)	272 (56%)
T2	32 (18%)	89 (23%)	69 (14%)
T3	83 (47%)	153 (38%)	138 (29%)
T4	4 (2%)	0	5 (1%)
Low*	47 (27%)	78 (20%)	159 (33%)
Intermediate*	119 (67%)	279 (70%)	255 (53%)
High*	11 (6%)	42 (10%)	70 (14%)

*: Risk groups are determined according to the simplefied clinical algorithm.

A concordance index was calculated to determine the predictive ability of the algorithm using the method introduced by Harrell et al. [10]. All pairs of patients from distinct risk groups were considered where one member of the pair was known to have survived longer than the other patient. The pair was considered concordant with respect to the staging if the patient who lived longer was in the lower risk group. The concordance index is the percent of pairs that are concordant.

A Cox model was used to determine the hazard ratios for the 3 centers considering the 3 risk groups to be labeled as low, intermediate, and high-risk. Data was analyzed using Stata 7.0 (College Station, TX).

Table 2
estimated disease specific survival rates according to risk group in patients with localized disease

Risk group	*Survival*	*St Radboud*	*MDA*	*UCLA*
Low	1-year	98%	98%	100%
	3-year	94%	98%	95%
	5-year	94%	92%	93%
Intermediate	1-year	91%	97%	97%
	3-year	77%	85%	87%
	5-year	65%	73%	78%
High	1-year	73%	80%	81%
	3-year	44%	52%	58%
	5-year	40%	30%	48%

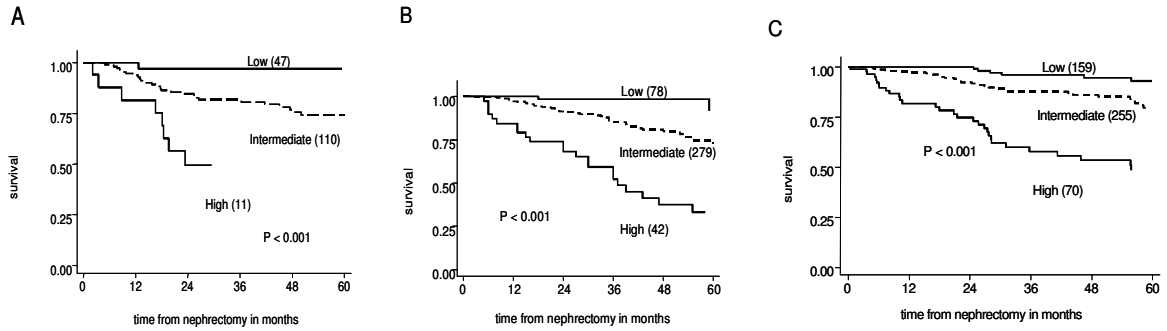
Risk groups are determined according to the simplefied clinical algorithm

2.4 Results

Table 1 lists the patient characteristics for the evaluated population with regards to sex distribution, Fuhrman grade, ECOG PS, TNM stage, and T stage distribution. Median follow-up for each of the 3 groups was 63 (SR), 32 (MDA), and 33 (UCLA) months. In addition, the table also depicts the number of patients that are in each of the risk groups from each of the respective institutions.

The decision box shown in appendix A can be used to determine the risk group of the patient; this is done by starting at the top of the decision box and progressing downward through the algorithm based on the patient's 1997 tumor stage, Fuhrman grade, and then ECOG PS. After the appropriate risk group has been determined, the patient's prognosis is then determined by looking up the outcomes in table 2.

Figure 1
Kaplan-Meier curves for the patients from each of the respective institutions.



A, Kaplan-Meier curves for 177 patients from the University Medical Center Nijmegen with localized renal cell carcinoma. Patients are stratified into 3 distinct survival curves based on low, intermediate, and high-risk characteristics. B, Kaplan-Meier curves for 399 patients from MD Anderson with localized renal cell carcinoma. Patients are stratified into 3 distinct survival curves based on low, intermediate, and high-risk characteristics. C, Kaplan-Meier curves for 484 patients from UCLA with localized renal cell carcinoma. Patients are stratified into 3 distinct survival curves based on low, intermediate, and high-risk characteristics.

In order for a staging system to be applied and validated in a center, it is not necessary that the survival curves be identical, but only that they result in clearly separated survivorship curves at each respective center. Figures 1a, 1b, and 1c depict the Kaplan-Meier curves for patients with localized disease for the 3 risk group categories from each of the respective institutions. The stratification trend across the 3 risk group categories for localized disease patients from each of the institutions was statistically significant for disease-specific survival (DSS) ($p < 0.001$). It is clear from each of the graphs that 3 distinctly separate survival curves result from use of the clinical algorithm for patients from each of the respective institutions.

For a comparison between the centers, the predicted 1, 3, and 5-year disease-specific survivals (DSS) for each of the 3 groups of patients stratified according to their risk group categories are listed in table 2. Estimated 5-year DSS for low-risk patients are 94%, 92%, and 93% for the SR, MDA, and UCLA patients, respectively. For the intermediate-risk patients, the rates are 65%, 73%, and 78% for SR, MDA, and UCLA, respectively. High-risk patients have estimated 5-year DSS rates of 40% (SR), 30% (MDA), and 48% (UCLA).

A concordance index was calculated to determine how accurate the clinical algorithm was at placing patients into the correct risk group. The concordance indices for each of the respective institutions were 79% (SR), 86% (MDA), and 83% (UCLA).Finally, hazard ratios and 95% confidence intervals for each of the 3 institutions were determined to further compare the relationship of the 3 risk groups within each institution. Patients in the SR group had a hazard ratio of 2.54 (1.54 - 4.19); in other words, a patient in the intermediate-risk group had a 2.54 times likelihood of dying from RCC when compared to a patient in the low-risk group. By comparison, the hazard ratio for MDA patients was 4.39 (2.93 - 6.59), while the ratio for UCLA patients was 3.15 (2.23 - 4.45).

2.5 Discussion

This clinical algorithm provides valuable information regarding survival after resection of localized RCC by stratifying patients into low, intermediate, and high-risk groups. As a result, this algorithm accounts for the interaction of 3 well-accepted variables that affect survival. This algorithm is easy to use and as illustrated in figures 1a, 1b, and 1c, provides statistically significant stratification of patients into 3 risk groups and outcomes. Staging systems differ from nomogram models in that the latter predicts different survivorship outcomes for individual patients. The staging of patients may be useful for evaluating clinical outcome, directing therapy, assessing patient response to treatment, determining eligibility for entry into clinical studies, and for interpreting results of clinical trials.

For several decades, published reports have emphasized the prognostic value of multiple factors for RCC such as stage, grade, and ECOG PS. The clinical outcome algorithm used in this study was used to generate 3 distinct risk groups applicable to an updated UCLA database and also to two other institutions by combining the 3 prognostic factors listed above.

This system allows physicians to confidently separate patients who undergo nephrectomy for localized disease into different risk groups. Information derived from the algorithm could be used to determine whether a patient in the high-risk group should receive either adjuvant immunotherapy or be enrolled into clinical trials. Patients in the high-risk group have 5-year DSS estimates of 40% (SR), 30% (MDA), and 48% (UCLA), suggesting that nephrectomy alone may not be enough.

By comparison, low-risk patients from each of the respective institutions have similar 5-year DSS estimates of 94% (SR), 92% (MDA), and 93% (UCLA). Patients in the low-risk group may require less rigorous surveillance compared to their intermediate-risk or high-risk counter-parts. This would likely decrease the number of computerized tomography scans, chest radiographs, and amount of blood work performed in the low-risk group. In turn, reductions in potential nephrotoxic and allergic reactions to dye contrast would result; in addition, decreased health care costs attributed to decreased surveillance testing would also result.

Other outcome models have been previously reported to predict survival in patients presenting with localized disease and metastatic disease. In 1988, Elson et al developed a scoring system to determine prognosis for patients with advanced RCC that stratified patients into five groups based on ECOG PS, time from diagnosis to metastasis, weight loss, prior chemotherapy, and number of metastatic sites [11]. In 1995, Mani et al found that 5 prognostic factors (ECOG PS, sarcomatoid histology, bone metastasis, weight loss, and absence of nephrectomy) could also be combined to form low, intermediate, and high-risk groups [12]. More recently, Memorial

Sloan-Kettering developed a model for patients with metastatic RCC that identified 5 different pretreatment features as important prognostic factors as determined by multivariate analysis. These factors included low Karnofsky score, anemia (hemoglobin < normal), high serum lactate dehydrogenase (> 1.5 times normal), hypercalcemia (> 10 mg/dl), and absence of nephrectomy [13].

As significant as the above prognostic models are for predicting outcome for patients with metastatic RCC, there remains a need for predicting clinical behavior after resection of localized RCC. In 2001, Kattan introduced a postoperative nomogram that assigns points based on histology, tumor size, 1997 P stage, and whether a patient presented with symptoms. The Kattan nomogram was designed to predict the 5-year probability of recurrence amongst patients who undergo resection for localized RCC and assigns points based on the 4 factors listed above. It does not offer prognostic capabilities for predicting survival. The purpose of nomograms such as this is different than the subdivision of patients into a small number of subgroups.

The SSIGN (Stage, SIze, Grade, Necrosis) recently introduced by the Mayo Clinic [5] uses 1997 TNM stage, tumor size > 5.0 cm, Fuhrman grade, and evidence of necrosis to stratify patients based on a point system as well. With a mean follow-up of 9.7 years, the SSIGN system stratifies patients into 10 survival curves.

The current algorithm used in this study is designed to separate patients into a small number of risk groups. It combines 3 well-known prognostic factors into an easy-to-use decision box (appendix) and then allows physicians to advise patients regarding 5-year survivals based on 3 distinct survival curves. External data from 2 different institutions was used to validate this algorithm, (figure 1). The similarity of the high concordance indices seen for each of the 3 institutions suggests that this algorithm may be applicable to other external databases. In addition, the survivorship curves for the 3 respective institutions demonstrate that each of the 3 subsets of patients within each institution can stratify survival for patients with localized disease into 3 distinct risk groups.

Limitations of this study include its retrospective nature and attendant issues routinely associated with such analyses. Other limitations of this study include the lack of central pathology, leading to concerns regarding interobserver reproducibility of grading. However, personal communication with pathologists from each of the 3 respective institutions confirmed that the same criteria proposed by Fuhrman in 1982 were used at all three institutions. Finally, similar to the other models described, the UCLA algorithm depends on postoperative variables, thereby limiting its utility as a preoperative counseling tool.

In conclusion, a clinical algorithm using 3 well-known prognostic factors can be used to predict survival and stratify patients undergoing nephrectomy for localized disease into 3 risk groups. External data from 2 outside institutions suggest the validity of this algorithm with a high concordance index. These risk categories can be used in clinical trial design and interpretation as well as in clinical management areas such as surveillance.

Appendix
Decision box to determine the appropriate risk category of patients with localized RCC

T Stage	1				2	3				4
Fuhrman Grade	1-2		3-4		⇓	1		>1		⇓
ECOG PS	0	≥1	0	≥1		0	≥1	0	≥1	
Risk Group	Low	Intermediate				Intermediate			High	

To obtain a risk group, begin at the top of the decision box and progress downward using patient 1997 American Joint Committee on Cancer T stage, Fuhrman grade and ECOG performance status at diagnosis.

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CHAPTER 3

VACCINATION OF PATIENTS WITH METASTATIC RENAL CELL CARCINOMA WITH AUTOLOGOUS DENDRITIC CELLS PULSED WITH AUTOLOGOUS TUMOR ANTIGENS IN COMBINATION WITH INTERLEUKINE-2: A PHASE I STUDY

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3.1 Abstract

Background: Dendritic cells (DC) have been recognized as the most potent antigen presenting cells (APC) of the immune system and clinical trials with DC presenting tumor antigens have shown promising results. We have previously shown that DC pulsed with autologous tumor lysates derived from renal cancer can activate tumor specific cytotoxic T-lymphocytes. Therefore, we performed a phase I study in patients with metastatic renal cell carcinoma, using autologous DC loaded with autologous tumor lysate as a vaccine. The treatment was combined with a low-dose IL-2 therapy, as this has shown benefit in DC based therapies.

Methods: DC were isolated from peripheral blood mononuclear cells (PBMC) of 12 patients. The adherent PBMC were cultured in the presence of IL-4 and GM-CSF. Following seven days of culture, the immature DC (iDC) were pulsed with the autologous tumor lysate derived from tumornephrectomy specimens. Three vaccinations were given at two-weekly intervals as intradermal single deposit injections. After each vaccination IL-2 was given to the patients for 5 consecutive days. In six patients, keyhole-limpet hemocyanin (KLH) was added to the DC culture medium after four days as an immunogenic marker. Before and after the vaccinations immunological and clinical monitoring was performed.

Results: In all patients a primary tumor cell culture could be established and sufficient amounts of tumor cells and tumor protein were obtained for vaccination purposes. We were able to generate iDC. The phenotype of the DC for the first vaccination was in general dissimilar from the phenotype of the DC for the last vaccination: most notably the number of CD14 positive cells increased. Ovalbumin uptake remained high, substantiating high uptake capacity, underlining that these cells were still functional, immature DC. Proliferative responses against KLH were observed, whereas proliferative responses against TuLy were absent. Humoral responses against TuLy or KLH were absent. Clinical responses were not observed.

Discussion: This study shows the feasibility of a completely autologous DC and tissue culture methodology for the generation of tumor lysate pulsed DC. The vaccine, consisting of iDC pulsed with autologous tumor lysate and KLH, was able to elicit anti-KLH responses, indicating the ability of the injected DC to mount an immunological response. However, no cellular, humoral or clinical anti-tumor responses were induced, suggesting that the vaccination strategy with immature DC has little benefit for patients with advanced RCC.

3.2 Introduction

Renal cell carcinoma (RCC) is a relatively rare disease, comprising about 2% of all malignancies [1, 2]. Approximately one-third of the patients present with metastatic disease (mRCC) and after radical nephrectomy with curative intent, about 30% of patients develop recurrent disease. mRCC does not respond to chemotherapy or radiotherapy [2]. Presently, interleukin-2 (IL-2) [3] and/or interferon-alpha (IFN-a) [4] are the standard therapies for progressive mRCC. However, the response rates are low (20%), with complete response rates of only 6% [3]. Thus, once metastasized, the prognosis of mRCC patients is poor. One of the major reasons for the ineffectivity of the standard immunotherapy approach might be lack of specificity of the agents given. Therefore new directions towards a more specific anti-tumor therapy-strategy is of utmost importance.

Dendritic cells (DC) have been recognized as the most potent antigen presenting cells (APC) of the immune system [5, 6]. The possibility to generate significant numbers of DC in vitro with functional characteristics of potent APC [7, 8], has lead to the initiation of numerous clinical trials exploring the efficacy of DC based vaccines, particularly after loading with peptides derived from tumor-associated antigens. Several studies have shown the feasibility of DC-based vaccinations, e.g., in B-cell lymphoma [9], melanoma [10], RCC [11, 12], and prostate carcinoma [13]. Up to 10^7 cells per vaccination have been used without any adverse side effects or toxicity when administered intravenously or subcutaneously (s.c.).

In a previous study we have shown that (tumor) specific cytotoxic T-lymphocytes (CTL) can be induced by autologous DC pulsed with autologous tumor lysates (TuLy) in vitro [7]. Various studies have described the superiority of the combination of DC-based vaccines and low-dose IL-2 [14, 15]. Based on these observations we performed a phase-I study to assess the feasibility of vaccination with autologous DC loaded with autologous tumor lysate in combination with IL-2 in patients with mRCC.

3.3 Patients & Methods

Twelve patients with mRCC with their primary renal tumor in place were enrolled in this study. Baseline clinical data of the patients were acquired before vaccination. Inclusion criteria were WHO performance status 0-2, over 18 years of age and capable of giving written informed consent. Patients with CNS metastases, allergic diathesis, hypercalcemia, significant cardiac problems, leukocytes < 2.0 x 109/l, platelets < 50 x 109/l, abnormal liver or kidney function were excluded from the study.
The study was approved by the Ethical Board of the University Medical Center Nijmegen, The Netherlands, in accordance with the current version of the declaration of Helsinki.

Generation of dendritic cells.
DC were collected as described earlier [16]. In short, 1 week before vaccination fresh peripheral blood mononuclear cells (PBMC) were obtained by differential centrifugation of autologous heparin blood on Ficoll-Paque R plus gradients (Pharmacia Biotech, Alameda, CA, USA). Cells were washed extensively in PBS (NPBI, the Netherlands) and suspended in 8 ml XVIVO (Biowhittaker, Walkersville, MD, USA) supplemented with 2% heat-inactivated autologous serum. PBMC were plated in 25 cm2 flasks (2.5-5 x 106 cells/ml) and incubated at 37^{O}C for 60 minutes. The non-adherent cells were aspirated and discarded. The adherent cells were gently rinsed with XVIVO (Biowhittaker) and cultured in XVIVO (Biowhittaker) supplemented with 10% autologous serum, 800 U/ml human recombinant Granulocyte Monocyte Colony Stimulating Factor (GM-CSF) and 1000 U/ml human recombinant Interleukin-4 (IL-4) (Schering-Plough, Kennilworth NJ, USA). After 7 days, all cells in suspension and loosely adherent cells were collected (see below).
In six patients, DC were pulsed with 70 ug Keyhole Limpet Hemocyanine (KLH)/flask 4 days after initiation of every DC culture. Three days thereafter all cells in suspension and loosely adherent cells were collected (see below).

Phenotyping of dendritic cells.
Cells were labeled with anti-CD14-FITC (DAKO, Marseille, France), anti-CD80-FITC, anti-CD40-PE, anti-CD83-PE (Immunotech, Westbrook, ME, USA) and anti-CD86-PE (Pharmingen, San Diego, CA, USA), respectively. After centrifugation and washing with PBS the cells were fixed in 1% paraformaldehyde/PBS and analyzed by FACS analysis (Beckman Coulter, Inc., Fullerton, CA, USA).

Preparation of the tumorlysate (TuLy).

Immediately after tumornephrectomy, in the operation theater, approximately 30g tumor tissue was minced and transferred to digestion medium containing 1 mg/ml Collagenase Type IV (Sigma, St Louis, MO, USA), 0.1 mg/ml Hyaluronidase Type V (Sigma), 30 U Deoxyribonuclease I Type IV (Sigma), 100U/ml Penicillin and 100ug/ml Streptomycin. After overnight digestion at room temperature the suspension was sieved over a 200 um mesh to remove the undigested fragments. This cell suspension was washed twice with PBS, applied on a Ficoll-Paque R plus gradient and centrifuged. The cells at the interphase were collected, washed three times with PBS and cultured in 75 cm2 flasks (0,5 x 10^6 cells/ml, 10 ml per flask) in RPMI 1640 supplemented with 100U/ml Penicillin, 100ug/ml Streptomycin, 2 mM Glutamine (complete medium), and 10% heat-inactivated autologous serum. After overnight adherence the medium was changed. After the cells had reached 70% confluence, the tumor cells were harvested by trypsinisation. Cells were resuspended in 5 ml PBS, irradiated (30 Gy), washed with PBS and resuspended in 2 ml PBS. Thereafter the cells were subjected to three freeze/thaw cycles, the protein concentration was determined (BioRad Laboratories B.V., Veenendaal, The Netherlands) and the obtained TuLy was aliquoted.

DC pulsed with TuLy.

TuLy (30ug/1 x 10^6 DC) was added to the DC (2-5 x 10^6 cells/ml) under serum-free conditions, and the DC were incubated for 30 minutes at room temperature. Thereafter an equal volume of complete medium containing 20% autologous serum was added, and DC were incubated for 3 hours at 37^{o}C. Subsequently, the cells were centrifuged and resuspended in 0.2 ml saline for intradermal injection.

Vaccination Schedule.

Vaccination started 3-5 weeks after the tumornephrectomy. DC were generated from freshly obtained PBMC isolated 1 week before every vaccination. Three vaccinations were given at two-week intervals as intradermal single deposit injections. Patients did not receive fixed amounts of DC, but rather the total amount of DC isolated. Sterility of all vaccines was determined before administration. After each vaccination patients received IL-2 (Proleukin® (Chiron, Emeryville, CA, USA)) 9x10^6U/day subcutaneous for 5 consecutive days by self-injection.

Clinical evaluation and follow-up.

Delayed type hypersensitivity (DTH) skin tests were performed at day 0 and day 42 (Multi Test CMI, Aventis Pasteur SA Lyon, France). After each vaccination patients were evaluated

Table 1
Patient characteristics

Patient	*Metastasis*	*pT*	*Grade*	*Karnofsky*	*Evaluation*
1	Lung	3a	IV	90%	SD 3.5 m*
2	Liver, bone	3a	III	80%	PD*
3	Lung, lymphoid organs	3b	III	100%	SD 5.0 m*
4	Lymphoid organs	3b	III	100%	SD 18.0 m
5	Lung, lymphoid organs	3a	III	90%	SD 4.0 m**
6	Lung, lymphoid organs	3a	II	100%	SD 3.0 m
7	Lung	3a	II	100%	PD
8	Lung	2	II	90%	SD 18+m
9	Lung, adrenal gland	3a	III	100%	PD
10	Bone, lymphoid organs	3b	III	100%	SD 12m
11	Lung	3b	II	100%	SD 16+m
12	Lung, bone, lymphoid organs	3a	III	90%	PD*

*: Died of disease **: Non-disease related death.
pT: Pathological stage; PD: progressive disease; SD: Stable disease.

according to the WHO Toxicity Criteria Scale. Baseline evaluation of tumor burden was assessed before the tumor nephrectomy. Three weeks after completion of three vaccination cycles, patients were evaluated by radiographic methods. Patients were followed at a 6 weeks interval until disease progression.

T-cell proliferation assay.

PBMC were stimulated with KLH, TuLy, Candida Albicans, Tetanus toxoid (4 ug/2 x 10^5 PBMC) or IL-2 (20 IU/ml) in complete medium with 10% human AB serum (Biowhittaker). On day 6 the cells were pulsed with ^{3}H-thymidine for 16 hours and harvested. Experiments were performed in triplicate. To cirvumvent assay-to-assay variation, all samples of one patient were analyzed simultaneously. Data were expressed as stimulation index (SI), calculated as:
SI = counts per minute after vaccination / counts per minute at study entry

IFN-Gamma secretion ELISA.

ELISA plates (Falcon, Becton-Dickinson, San Jose, CA, USA) were coated with 100 ml anti-human IFN-gamma monoclonal antibody (mAb, (Endogen, St Woburn, MA, USA) at 0,75ug/ml PBS overnight at 25^{O}C. After blocking remaining protein binding sites for 1 hour at 25^{O}C with 1% gelatin/PBS (Sigma) plates were washed with PBS/(0.05%) Tween (Sigma). Tissue culture supernatant of PBMC stimulated for 6 days were added and incubated at 25^{O}C for 1 hour. Plates were washed and incubated with biotin labeled anti-human IFN-gamma mAb

(Endogen), 0.2 ug/ml in 1% gelatin/PBS for 1 hour at 25^{o}C. After washing, plates were incubated with horseradish peroxidase labeled streptavidin (CLB, Amsterdam, Netherlands) diluted 1:15.000 in 1% gelatin/PBS for 30 minutes at 25^{o}C. Plates were developed with 3,'3,5,'5-tetramethanolbenzidine (TMB) in 0.1 M acetatebuffer pH 5.5/ 0.03% H_2O_2 for 20 minutes at room temperature in the dark. Absorbance was measured at 450 nm.

Analysis of humoral immune response.
ELISA plates (Falcon, Becton-Dickinson) were coated with 100ul KLH (20 ug/ml) or TuLy (1 ug/ml) overnight at 4^{o}C. Plates were blocked for 2 hours with 1% gelatin/PBS at 25^{o}C. After washing with 0.05% PBS/Tween, plates were incubated with serial dilutions of patient sera obtained before, during and after treatment, for 1 hour at 25^{o}C. After washing, plates were incubated with peroxidase labeled goat-antihuman Ig (Sigma) in 1% gelatin/PBS. Plates were developed, and absorbance was measured as previously described.

3.4 Results

Between February 1999 and February 2000, 12 patients of the Kidney Cancer Clinic of the Urology and Medical Oncology Department of the University Medical Center Nijmegen, The Netherlands, were included in the protocol. All patients signed an informed consent before study entry. The characteristics of the 12 patients are listed in table 1. Enrolled were 8 men and 4 women with a median age of 55 years (range: 43-69). Performance state was > 80% according to Karnofsky. Pathological assessment of the primary RCC tumors showed that all patients but one (Pt 11: chromophilic) had RCC tumors of the clear-cell type.

Vaccine preparation.
A primary cell culture could be established from all radical tumornephrectomy specimens and sufficient amounts of tumor cells (mean: 2.6x10^{8}, range: 0.4-9.7x10^{8}) and tumor protein (mean: 3.6 mg, range: 1.4-7.2 mg) were obtained for vaccination purposes. Phenotypic analysis showed that the cells harvested were predominantly cytokeratin positive, confirming outgrowth of tumor cells and minimal contamination of fibroblasts. In general, tumor cells were harvested 3 weeks after establishment of the primary culture for TuLy preparation.
The yield of PBMC and the characteristics of the generated DC are listed in tables 2 and 3.
In this protocol, DC were generated from freshly obtained PBMC, isolated one week before every vaccination. In general, the yields of PBMC and, to a lesser extend, the number of iso-

Table 2
Average yield of peripheral blood mononuclear cells and dendritic cells of the 12 patients.

		Vaccination 1	Vaccination 2	Vaccination 3
Yield of PBMC	($*10^6$)	78 (34 - 140)	99 (65 - 133)	131 (80 - 225)
Yield of PBMC/ml	($*10^6$)	0.8 (0.4 - 1.4)	1.0 (0.7 - 1.2)	1.4 (0.9 - 2.4)
Yield of DC	($*10^6$)	9.1 (2.4 - 17.0)	15 (4- 34)	14.6 (4.0 – 36.0)
Yield of DC/ml	($*10^6$)	0.09 (0.03 - 0.19)	0.15 (0.04 - 0.34)	0.15 (0.04 - 0.36)

lated DC, increased at subsequent blood drawings. The yield of PBMC and DC increased from an average of 78 x 10^6/ 9 x 10^6 cells at the first blood draw to an average of 131 x 10^6/ 15 x 10^6 cells respectively at the third blood draw (table 2). In most testcultures we were able to generate DC of an immature phenotype ($CD14^{low}$, $CD86^{high}$, $CD80^{low}$, $CD40^{high}$, $CD83^{low}$: fig. 1). The phenotype of the DC for the first vaccination was, in general, dissimilar from the phenotype of the DC for the last vaccination. CD86 and CD40 remained high and CD83 remained low, but the number of CD14 positive cells increased (table 3).

In selected cases, CD11c phenotype was determined, and, under the culture conditions used in this trial, all cells were $CD11c^+$. Thus, CD11c did not provide extra information on the presence of non-DC cells or DC phenotype. Despite this unusual phenotype, these DC still exhibited high uptake capacity, as determined by FITC-ovalbumin uptake, substantiating that these cells were still functional, immature DC.

Immunological monitoring.

Analysis of proliferative responses of PBMC obtained at various time points during and after completion of the vaccination did not reveal any measurable response against TuLy in any of the patients. In contrast, almost all patients showed strong proliferative responses against Candida Albicans or Tetanus toxoid, two recall antigens. Moderate proliferative responses of PBMC against KLH were observed in patients vaccinated with DC loaded with KLH and TuLy translating to moderate stimulation indices (fig. 2). In concordance with the T-cell proliferation, KLH stimulation resulted in a low specific IFN-gamma secretion (data not shown). Humoral responses against TuLy or KLH were absent.

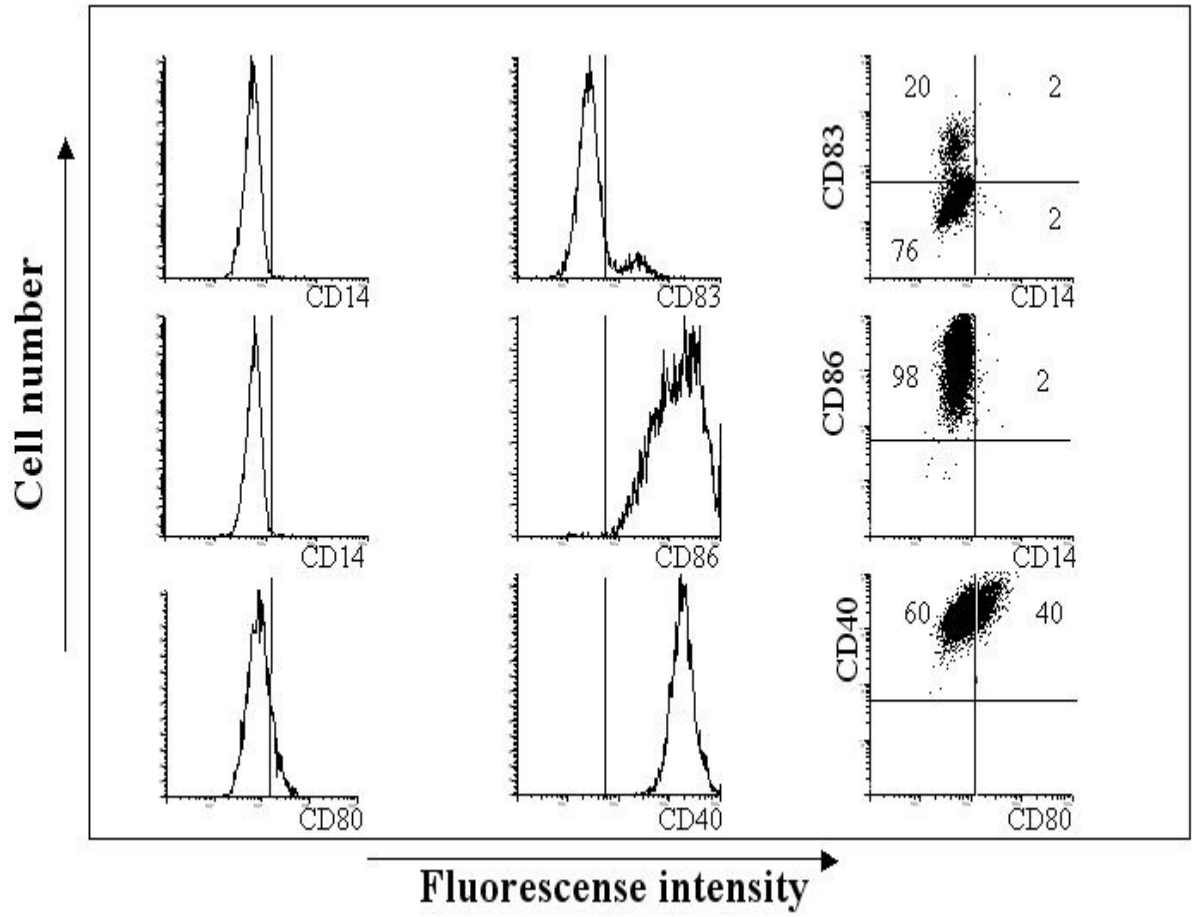
Before the vaccinations, nine of the twelve patients had a DTH response against more than one

Table 3
Dendritic cell phenotyping of a representative patient.

	CD14+	*CD86+*	*CD40+*	*CD80+*	*CD83+*
testculture	2	99	99	7	10
1e vaccine	1	94	93	7	11
2e vaccine	13	93	98	4	2
3e vaccine	30	95	98	8	6

Numbers represent the percentage of positive cells.

Figure 1
Phenotype of a7-day autologous immature dendritic cell test culture



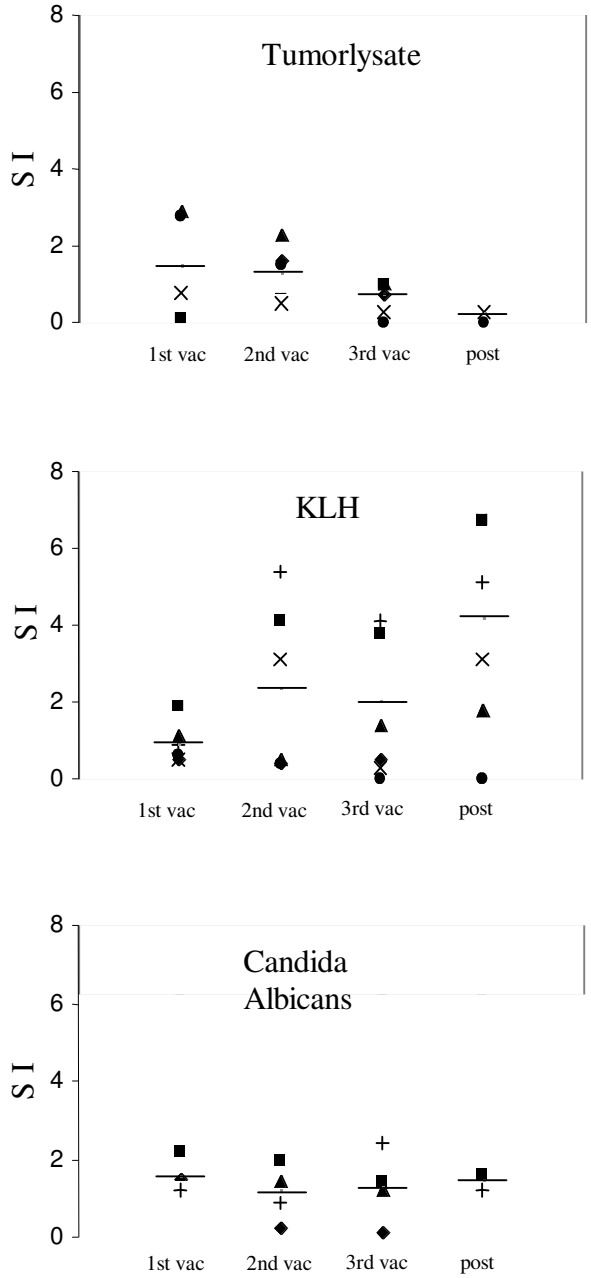
The DC were stained with antibodies specific for CD14, CD80, CD40, CD83 and CD86 and analyzed on a flow cytometer. Consistent with the phenotype of immature DC, the cells expressed low levels of CD14 and CD83 and high levels of CD86 and CD 40. The percentage of cells expressing particular cell surface markers is shown in appropriate quadrants.

of the eight recall antigens used. One patient developed a DTH response against a recall antigen after the vaccination, whereas there was no response prior to the vaccinations. In five patients the response to the number of recall antigens increased. None of the patients showed a DTH response to the tumor lysate before or after the vaccinations.

Clinical observation.

The treatment was well tolerated, and no significant side effects were seen. Transient erythema and/or induration at the site of the injection was observed in five patients. Patients experienced typical side effects of low-dose s.c. IL-2 treatment: moderate fever, chills and fatigue. Side effects were not enhanced by the addition of DC vaccination. Toxicity was < grade 2, and did not lead to treatment discontinuation. Four patients showed progressive disease at the first clinical evaluation after treatment (3 months). In one of these patients pulmonary metastases

Figure 2:
Analysis of proliferative responses of peripheral blood mononuclear cells (PBMC)



Analysis of proliferative responses of peripheral blood mononuclear cells (PBMC) at various time points during after completion of the vaccination. Two hundred thousand PBMC were stimulated for 6 days with TuLy, KLH or Candida Albicans, respectively. On the 6th day, the cells were pulsed with [3H]thymidine for 16 hours, after which the proliferation was measured. Data were expressed as stimulation index (SI), i.e. [3H] counts per minute after vaccination/[3H] counts per minute at study entry; lines represent mean SI. Each marker represents an individual patient; 1st vac (2 weeks after 1st vaccination); 2nd vac (4 weeks after 1st vaccination); 3rd vac (6 weeks after 1st vaccination); post (3 months after last vaccination)

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were removed (Pt 7). This patiënt is still free of disease. In six patients disease progression was observed after a mean period of stable disease of 7.6 months (range 3.0 - 18.0 months). Two patients (Pt 8 & 11) are still stable with a mean period of 17.0 months (range 16+ - 18+ months). In one patient pulmonary metastases were removed. This patient is now still free of measurable lesions. One non-disease related death occurred in a patient with stable disease (6 months). No partial or complete responses were seen (table 1).

3.5 Discussion

To study the potential role of DC-TuLy-based vaccines as therapy for patients with advanced RCC we performed a phase-I clinical trial with DC loaded with tumor lysate in combination with IL-2. The influences of allogeneic or autologous culture conditions of DC and the use of allogeneic or autologous tumor tissue on the efficacy of DC vaccination are unknown. Therfore, we choose a completely autologous setting, Because this enables appreciation of immune responses without interference of , e.g., bystander effects, reactivity against minor anigens, etc., through the use of allogeneic cells. We have previously shown the feasibility to generate autologous DC able to induce CTL with strong autologous tumor cell-killing capacity in vitro [7]. Furthermore, Shimizu et al. [14], and Rosenberg et al. [15] demonstrated that low dose IL-2 can potentiate the anti-tumor effect of TuLy pulsed DC in vivo and Höltl et al [11], showed induction of cellular and humoral immune responses in patients with advanced RCC after vaccination with mature dendritic cells pulsed with TuLy. The latter was a clean illustration that immunogenic structures might be present on autologous RCC. Our use of immature DC was based on the observation that antigen-uptake capacity of immature DC is superior over uptake capaciry of mature DC [17]. In view of our pulsing strategy, maximal uptake capacity seemed to be essential. These observations formed the foundation of this phase I clinical trial with autologous, immature DC, pulsed with autologous TuLy in combination with a low dose IL-2 regimen similar to Rosenberg et al. [15]. Recently, Fong et al. [18] showed clear evidence that intradermally injected DC might mature in vivo , i.e., the use of immature DC does not appear disavantageous.

In all patients and for all vaccinations, significant numbers of DC were obtained. Not unexpectedly, administration of low dose IL-2 led to increased numbers of PBMC harvested. In parallel, we observed a similar increase in DC numbers generated. It is well documented that IL-2 administration results in T cell proliferation [19], but the influence of biological response modifiers on the development of DC from PBMC still needs to be clarified. In our trial the

phenotypic profile of the DC cultured at vaccine initiation differed from the phenotype of DC cultured for the last vaccination (table 3). This may be the result of the application of IL-2 and its effect in vivo. This is in line with the observations of Merad et al [20] who investigated the phenotypic modulations of DC during the treatment with IL-2/IFN-alpha or IL-12 in patients with mRCC. They found that PBMC from these patients maintained the ability to differentiate into functional DC, without selective quantitative or qualitative advantage [20]. Despite this somewhat different phenotype, protein-uptake experiments demonstrated high protein uptake capacirt, confirming that these cells were still functional, immature DC. Because it is unclear whether the number of injected DC correlates with immunologic and clinical responses, Patients received the entire amount of cultured DC generated.

In melanoma, many vaccination trials have been performed with peptide loaded DC (reviewed in [21]). This was based on the isolation of CTL directed against various melanoma-associated antigens from PBMC of patients with melanoma, suggesting that these antigens can serve as CTL targets. In human RCC, however, only a few specific tumor antigens, such as G250 and RAGE [22, 23], are known. RAGE, being expressed in a minor percentage of RCC is, therefore, a suboptimal target. G250 is expressed in the majority of RCC, and an HLA-A2.1-restricted epitope recognized by CTL is present in this protein [24]. However, G250-specific CTL could not be isolated from TIL of patients with RCC [22]. Thus, it is unclear whether this antigen is a suitable T cell target. In the absence of clearly defined potentially immunogenic peptides, we pursued vaccination with tumor-extract pulsed DC. This strategy can lead to dramatic anti-tumor responses (e.g. [25]), and circumvents the requirement of using identified tumor specific or tumor-associated antigens. Furthermore, multi-antigen loaded DC are likely to give poly- or oligoclonal expansion of T-cells, which might have an enhanced antitumor effect in comparison to peptide-pulsed DC. Nevertheless, despite the use of complete TuLy, no immunologic response was measurable in any of the patients. This is in contrast with the results of Höltl et al [11] who used intravenously infused mature DC pulsed with TuLy and KLH. These investigators were able to detect cellular and humoral responses against TuLy. It is possible that this difference is due to our use of immature DC.

In addition to the significance of IL-2 supplementation and pulsing with TuLy instead of well-defined peptides, the route of administration and maturation status are also of importance. Patients received intradermal injections based on reports that DC distribution to sites of lymphoid tissue is dramatically influenced by the route of administration and that intradermal injections appear to result in migration to regional lymph nodes and are qualitatively superior [18, 26]. TuLy was pulsed immediately before DC administration, taking advantage of the ability of immature DC to effectively capture antigens [17,27,28]. Theoretically, mature DC

might be preferable for the initiation of a powerful anti-tumor response. They may exhibit superior migration characteristics [29], although this has been disputed [30]. Additionally, mature DC are superior in antigen presentation and priming of naïve T cells [8], in particular because upregulation of MHC class I and II expression and co-stimulatory molecules occurs. Moreover, they acquire the ability to produce IL-12 and become potent initiators of both a primary and a secondary T-cell response [17]. However, Barratt-Boyes et al [30] documented that the state of maturation at the time of injection had no influence on the proportion of DC that localized to draining lymphoid organs, as labeled immature and mature DC were detected in equal numbers. These investigators concluded that in vitro maturation is not a requirement for migration of the DC from the injection site to lymphoid organs. They suggested that the use of immature DC loaded with TuLy undergoing natural maturation in vivo might be preferred over in vitro maturation [30]. In view of the superior protein uptake capacity, similar migration characteristics of immature DC over mature DC, the uncertainty of appropriate in vitro maturation methods, as well as evidence that intradermal injected immature DC can mature in vivo, immature DC were used. In our study, cellular immune responses against TuLy were not observed, whereas anti-KLH responses were moderate. In addition, humoral immune responses against TuLy and KLH were completely absent. Although this indicates that the administered immature DC were functionally capable of inducing only a minimal immune response, this response was much lower than the response described by Höltl et al. [11] who used intravenously infused mature DC pulsed with TuLy and KLH. In twelve patients with mRCC a strong cellular and humoral immune response against KLH waas detected and additionally, cellular and humoral responses were detected against TuLy, albeit at a lower level [11]. Thus, the maturation status of DC seems to be of utmost importance, and it appears preferable to pulse immature DC with TuLy followed by in vitro maturation for the induction of a powerful immune response.

Simons et. al. [31] used GM-CSF transduced autologous tumor cells to induce antitumor responses in patients with RCC. This study was based on extensive animal experimentation showing that vaccines consisting of GM-CSF transduced tumor cells were superior inducers of antitumor responses [32] possibly through eosinophils. Indeed marked infiltration ofeosinophils was observed in DTH sites of patients with RCC, and one patient showed a partial clinical response. the rationale of our DC study and the GM-CSF vaccine study are fundamentally different. Although the diversity of RCC antigens is similar, we have loaded DC with tumor proteins ex-vivo, whereas Simons et. al. [31] aimed at in vivo activation of APC. Different effector cells seem to be involved, and the circumstances of antigen presentation differ. Therefore these trials are difficult to compare.

Unfortunately, there is no consensus in the literature as to the appropriate/optimal vaccine dose. Tumor load, immunologic status, and tumor type are all parameters that are likely to vary between patints and influence the effects of DC vaccination. Therefore, in general, investigators tend to inject as many DC as possible, the strategy used here being that all DC harvested are injected, divided over several intradermal deposits. the biweekly vaccination schedule was realistic from a logical (patient) standpoint, as well as from an immunologic standpoint. In view of the lack of knowledge of (potential) CTL targets and their immunogenicity, little could be tuned with regards to the dosing and timing schedule.

To our knowledge, the sufficient dose of tumor cells/protein has not been determined in any comparable study. We based the amount of protein pulsed on DC on our earlier results where 30ug/10^6 DC was suffucient for the induction of CTL able to kill autologous RCC celss [7]. Clearly, this amount was insufficient for the induction of immune responses in the vaccinated patients with mRCC. It is unlikely that this failure is the consequence of the amount of pulsed protein. Remarkably, a pulse with 20 ug KLH/1x10^6 cells induced only a very moderate anti-KLH response, and, therefore, it is likely that the failure to induce anti-tumor immune responses is caused by the use of immature DC. This conclusion is supported by recent results of Jonuleit et al. [33] who reported that immature DC can induce the development of IL-10 producing non proliferating T-cells. Furthermore, these T-cells lost their ability to produce IFN-gamma, IL-2 and IL-4. Possibly, induction of such cells can explain why CTL responses against TuLy were absent, and responses against KLH were only moderate.

Despite the addition of IL-2 no objective clinical responses were observed. This contrasts to the results of Shimizu et al1 [14] and Rosenberg et al [15] who describe a positive effect of the combination of IL-2 with specific immune therapy. Nevertheless, in eight of twelve patients with mRCC, treated with surgery, DC vaccination and IL2, stabilization occurred. This stabilization might be the effect of vaccination. Recently, several investigators have also explored the use of DC based vaccines for patients with mRCC [11, 12]. Höltl et al. [11,34] used intravenously infused mature DC pulsed with TuLy with one out of four patients responding [11,34]. Gitlitz et al [35] treated mRCC patients with immature DC pulsed with TuLy/liposomes. In one patient changes in immunological parameters were observed, as well as a short-lived partial response. The poor clinical response rates showed that this potentially new treatment needs to be optimized. In this regard the study of Kugler et al. [12] who evaluated a vaccine using tumor cell/DC hybrids is of interest. These investigators used electrofused autologous tumor cells in combination with allogeneic DC, resulting in impressive (41%) clinical response rates. Interestingly, their results indicated that electrofused RCC/DC hybrids can elicit HLA restricted T cell responses with potent anti-tumor response.

In conclusion, the current study demonstrates that it is feasible to generate a DC-based vaccine in a completely autologous setting. The observation that this vaccine, consisting of immature DC pulsed with autologous TuLy and KLH in combination with IL-2 was able to elicit anti-KLH responses, showed that the injected DC were functional. Nevertheless, the immune responses were minor, and immune responses against tumor antigens were not observed, indicating that this vaccination strategy was suboptimal. Clinical benefit is minimal; emphasizing that further optimization is needed. Currently, trials investigating the effect of mature DC/ RCC hybrids in the treatment of renal cell carcinoma patients are in progress.

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CHAPTER 4

VACCINATION OF PATIENTS WITH PROGRESSIVE RENAL CELL CARCINOMA WITH CA9-PEPTIDE PULSED MATURE DENDRITIC CELLS

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4.1 Abstract

Introduction: Carbonic Anhydrase-IX$^{G250/MN}$ (CA9) is a renal cell carcinoma (RCC)-associated antigen ubiquitously expressed in the clear-cell subtype of RCC. Two CA9-derived peptides have been identified defining a CTL epitope and HLA-DR epitope respectively able to induce T cell responses in vitro. A phase-I clinical trial was performed with CA9-peptide loaded dendritic cells in patients with progressive, cytokine-refractory metastatic renal cell carcinoma to assess the safety, toxicity and induction of CA9-specific immunity.

Methods: Patients with objective progressive metastatic RCC received 5 vaccinations of mature dendritic cells (mDC) pulsed with the CA9-derived peptides and KLH. Peripheral blood was collected at regular intervals, delayed-type hypersensitivity (DTH) was tested at baseline and after the last vaccination and skin biopsies of positive DTH-sites were collected for immunomonitoring purposes. Patients were also monitored for clinical responses.

Results: No significant toxicity was observed. In all patients humoral responses were observed against KLH, as well as DTH conversion. Evaluation of biopsy material suggested increased influx of T-helper cells. In none of the immunomonitoring assays evidence for the induction of CA9-peptide specific immunity was observed. No clinical responses were observed.

Conclusion: The vaccination of DC-pulsed with KLH and two CA9 derived peptides was well tolerated. The lack of induction of CA9-peptide specific immune responses indicate that this particular peptide combination cannot induce peptide-specific immune responses.

4.2 Introduction

Patients with metastasized renal cell carcinoma (mRCC) have a poor prognosis. Radio- and chemotherapy are not effective [1,2]. Experimental treatments currently under investigation include anti-VEGF monoclonal antibody Bevacizumab, small molecule receptor tyrosine kinase inhibitor Sutent (SU11248), and targeting the Raf kinase pathway Sorafenib (BAY 43-9006) [3-6]. At present cytokine based therapy is the only registered treatment for mRCC with the ability to induce long-term responses, albeit in a minority of patients [7].
In view of the non-specific character of these cytokine regimens, methods to induce more specific immune responses against RCC are under investigation. Dendritic cells (DC) have been identified as the most potent antigen presenting cells of the immune system [8] and collective results of DC-based clinical trials have demonstrated the safety and feasibility of this approach [9,10]. In the development of effective DC-based vaccines knowledge of potentially immunogenic structures is of importance. In RCC, the RCC-associated antigen Carbonic Anhydrase-IX$^{G250/MN}$ (CA9) was identified [11]. CA9 is present in >95% of the clear-cell subtype of RCC. Moreover, no expression can be detected in the majority of normal tissues, including normal kidney tissue. Expression in other normal tissues is limited to large bile ducts and gastric epithelium [12]. Previous studies have identified a MHC class I and MHC class II CA9-derived peptide and confirmed their potential immunogenicity in vitro [13,14]. These findings, together with the high prevalence of CA9 in RCC appear to make these peptides potential candidates for peptide-pulsed DC vaccination to induce both CD4+ and CD8+ RCC specific T-cells. Therefore, we tested whether vaccination of CA9 peptide loaded DC of progressive mRCC patients resulted in CA9 specific immunity.

4.3 Patients & Methods

Patient Selection
Patients with cytokine refractory and progressive mRCC of the clear cell subtype were enrolled in the study protocol. Inclusion criteria were HLA-A2.1+ and HLA-DR+, WHO performance status 0-1, over 18 years of age and capable of giving written informed consent. Patients with CNS metastases, allergic diathesis, hypercalcemia, significant cardiac problems, leukocytes < 2.0 x 109/l, platelets < 50 x 109/l, abnormal liver or kidney function were excluded from the study. The study was approved by the Ethical Board of the University Medical Center Nijmegen, in accordance with the current version of the declaration of Helsinki. All patients

signed an informed consent form before entering the study protocol.

Study Design

Patients received 5 intradermal vaccinations in week 1, 2, 3, 6 and 12 respectively. Patients received an approximate one-fifth of the DC yield pulsed with the two peptides. Medical history was recorded at baseline and medical performance was recorded at every visit. Standard blood test, urine analysis and blood draw of peripheral blood for immunological monitoring was performed at baseline and weeks 4, 7, 13, and 16. Radiological assessment was performed 4 weeks after the last vaccination. Delayed type hypersensitivity (DTH) skin testing was performed one week before the first vaccination and at week 4 and 13. When redness and induration was induced, a 6mm skin biopsy was performed 48 hours after the DTH skin testing performed at week 13.

Carbonic Anhydrase-IX$^{G250/MN}$-derived peptides

HLA-A2.01-restriction (nonamer): 254-262 (CA9p254, HLSTAVARV)

HLA-DR-restriction (20-mer): 249-268 (CA9p249, IHVVHLSTAVARVDEALGR)

The CA9-derived peptides were manufactured, vialed and distributed by Clinalfa AG, Switzerland, under GMP conditions, approved for clinical use. The product was supplied as sterile, pyrogenfree lyophilized material aliquoted in 200 ug amounts. The purity of the peptide was >95% (HPLC).

Generation and Pulsing of Dendritic Cells

A concentrated leukocyte fraction was generated through leukapheresis, processing 6 liters of blood. Peripheral blood mononuclear cells (PBMC) were obtained by differential centrifugation of the leukocyte fraction on Ficoll-Paque R plus gradients (Pharmacia Biotech, Alameda, CA). Cells were washed extensively in PBS (NPBI, the Netherlands) and suspended in DC medium: XVIVO-15 (Biowhittaker, Walkersville, MD) supplemented with 2% heat-inactivated pooled human AB serum (HAB, Bloodbank Rivierenland, Nijmegen, The Netherlands). PBMC were plated in 225 cm^2 flasks ($10x10^6$ cells/ml, 30 ml/flask), and incubated at 37^oC/5%CO2 for 60 minutes. The non- and loosely adherent cells were aspirated. The adherent cells were cultured in complete DC culture-medium (DC medium supplemented with 800 U/ml human recombinant Granulocyte Monocyte Colony Stimulating Factor (GM-CSF) and 500 U/ml human recombinant Interleukin-4 (IL-4), both Schering-Plough, Kennilworth NJ)). At day 4, cells were transferred to 6-well plates in 2ml of complete DC culture-medium

($0.5x10^6$ cells/ml) and Keyhole Limpet Hemocyanine (KLH; Calbiochem, San Diego, CA, USA) was added (25 ug/ml). For monitoring purposes a small amount of DC were plated without the addition of KLH. The immature DC were matured at day 6 for 48 hours. One ml of complete DC culture medium was added to the 6-wells with the inclusion of tumor necrosis factor-alpha (TNF-alpha; Sigma Aldrich, Dorset, U.K.), final concentration 20ng/ml, and prostaglandin-E (PGE-2; Prostin E2, Pfizer, The Netherlands), final concentration 0.35 µg/ml. DC were harvested and frozen at -140^0C until use (10^7 cells/vial). On the vaccination day DC were thawed, resuspended in XVIVO-15, divided in two equal amounts and pulsed with CA9p254 or CA9p249 respectively. DC were pulsed for 90 minutes at 37 °C/5% CO^2 (75 µg/ml), fresh peptides were added (125 µg/ml), and DC were kept at room temperature for 90 minutes. After peptide pulsing DC were pooled and prepared for vaccination (final injection volume 400 µl).

DTH skin testing

DTH skin testing with (a) PBS, (b) KLH (5mg/injection), (c) the CA9p254 peptide and the (d) CA9p249 peptide (100mg peptide/injection) was performed at baseline. At week 4 and 13 DTH skin testing was repeated, with the addition of (e) unpulsed DC, and DC pulsed with (f) KLH, (g) KLH plus CA9p254 and (h) KLH plus CA9p249 (2.5x105 DC/injection).

At week 13, 2 days after the skin testing, a biopsy was taken of normal skin together with positive DTH sites. Biopsies were divided for immunohistochemical evaluation and expansion of DTH infiltrating lymphocytes (DIL). Tissue was cultured in RPMI 1640 supplemented with 5% HAB and recombinant IL-2 (100 U/ml) for 3-4 weeks. On day 7, 14 and 21 half of the medium was replaced by fresh IL-2 containing medium. At several time points during culture, supernatant was collected and stored at -20^oC. After 3-4 weeks of culture the cells were harvested and analyzed.

T-cell Stimulation.

Peripheral blood lymphocytes (PBL; 1 x 10^6/well of 24-wells plate) obtained at baseline and during the trial were cultured in complete RPMI medium supplemented with 5% HAB (Biowhittaker, Walkersville, MD), recombinant IL-2 (15 U/ml) and IL-7 (25 ng/ml). The cells were stimulated at a 10:1 ratio with unpulsed DC, DC pulsed with KLH or DC pulsed with CA9-derived peptides. Also non-stimulated PBL were evaluated. At day 2 half of the medium was replaced by IL-2 and IL-7 containing medium, at day 5 half of the medium was replaced by IL-2 containing medium. At days 7-14 PBL were re-stimulated as described above. At day 14 cells were harvested and analyzed. If sufficient DIL were harvested these cells were stimu

lated as described above.

Phenotype Analysis of DC, PBL & DIL

DC were labeled with anti-CD14-FITC (DAKO, Marseille, France) / anti-CD86-PE (Pharmingen, San Diego, CA), anti-CD80-FITC / anti-CD40-PE (both Immunotech, Westbrook, ME), anti-CD14-FITC / anti-CD83-PE (Immunotech, Westbrook, ME), anti-MHC class-I / anti-MHC class-II (both DAKO, Marseille, France). PBL and DIL were labeled with anti-CD-3-FITC (DAKO, Marseille, France) / anti-CD56-PE (BD Biosciences, Belgium), anti-CD4-FITC (Immunotech, Westbrook, ME) / anti-CD8-PE (DAKO, Marseille, France). After centrifugation and washing with PBS the cells were fixed in 1% paraformaldehyde/PBS and analyzed by FACS analysis (Beckman Coulter, Inc., Fullerton, CA).

Cytokine Profile

Supernatant of the T-cell stimulation assays (PBL and DIL) were collected at days 2 and 9 during the stimulation protocol. The secretion of cytokines into the supernatant was evaluated using a Bio-Plex Human Cytokine Th1/Th2 Panel (Bio-Rad, Veenendaal, The Netherlands) which includes antibody-conjugated beads, detection antibody and standards for simultaneous detection of IL-2, IL-4, IL-5, IL-10, IL-12p70, IL-13, GM-CSF, IFN-gamma and TNF-alpha. The plates were read on the Luminex 100 apparatus (BioRad)

Detection of CTL specific for CA9$^{MN/G250}$-derived peptides

Stimulated PBL and DIL were evaluated for the presence of peptide-specific CTL directed against CA9p254 by DimerX peptide presentation technology (BD Biosciences, Erembodegem, Belgium), involving passive peptide loading at peptide excess (overnight at 37^0C, 640x molecular excess) according to the manufacturer's instructions. In brief, PBL were resuspended in 0.5% BSA/PBS ($1x10^6$ cells/50 ml) and polyclonal human IgG was added to block non-specific binding of the DimerX or antibody reagents to surface Fc receptors. Peptide-loaded HLA-A2:Ig was added to the sample (2 mg/10^6 cells), incubated at 4 °C for 1 hour, and washed. Polyclonal human IgG was added (10 minutes room temperature) followed by the addition of PE-conjugated rat-anti-mouse IgG1 (clone A85-1, Becton-Dickinson, Alphen aan de Rijn, The Netherlands) and anti-human CD8-FITC (clone 42-8, Becton-Dickinson) for 30 minutes at room temperature. The cells were washed and analyzed by flow cytometry.

Analysis of humoral immune response.

ELISA plates were coated with 2mg KLH (20 mg/ml) or individual CA9 peptides (20µg/well) overnight at 4^{o}C. Plates were blocked for 2 hours with 1% gelatin/PBS at 25^{o}C. After washing with 0.05% PBS/Tween, plates were incubated with serial dilutions of patient sera obtained before, during and after treatment, for 1-2 hours at 25^{o}C (starting 1:100, 3-fold dilutions). After washing, plates were incubated with peroxidase conjugated goat-antihuman Ig (Sigma, 1: 2000) in 1% gelatin/PBS for 30 min at 25^{o}C. Plates were developed with 3,'3,5,'5-tetramethanolbenzidine (TMB) in 0.1 M acetatebuffer pH 5.5/ 0.03% H^2O^2 for 20 minutes at room temperature in the dark. Reactions were stopped with 2M H^2SO^4. Absorbance was measured at 450 nm.

Immunohistochemical staining of DTH skin biopsies

Cryosections (5µm) of tissue specimen were fixed for 10 minutes in ice-cold acetone. After washing with PBS the sections were incubated with anti-CD3-Alexa488; anti-CD4-Alexa488, anti-CD8-Alexa647 (Molecular Probes, Leiden, The Netherlands), anti-CD8-PE; anti-CD56-PE; anti-CD4-FITC; or anti-CD11c-PE (Immunotech, Westbrook, ME) for 30 minutes at room temperature. After repeated washing with PBS, slides were covered with fluorescent mounting medium (DAKO Cytomatics). Sections were analyzed with a fluorescence microscope or by confocal microscopy.

Detection of CA9 specific CTL in DTH biopsies was performed as decribed by de Vries et al.[15]. Briefly, cryosections (8µm) were air- dried and fixed for three minutes with 4 % paraformaldehyde and rinsed with PBS. The sections were blocked with 20 % normal goat serum and incubated with CA9-tetramer (Kindly provided by Dr. R.A. Willemsen, Rotterdam, The Netherlands) overnight at 4° C in 4% normal goat serum/PBS. After washing with PBS and fixation in paraformaldehyde for 20 minutes, sections were incubated for 30 minutes with rabbit-anti-streptavidine (1:800, Rockland, Gilbertsville, PA) with or without mouse-anti-CD8 (1:100, Pharmingen, San Diego, CA). The specific binding of tetramer was visualized using a polyclonal goat-anti- rabbit-Alexa 594 (Molecular Probes, The Netherlands) and CD8 was visualized using a polyclonal goat-anti-mouse- Alexa 488 (Molecular Probes).

Table 1
Yield of PBMC and DC

	Average	*Range*
Yield PBMC x10^8	24	10 – 50
Yield DC x 10^6	282	36 – 792
% DC / PBMC	13.4	2.6 – 31.7

Abbreviations: PBMC= peripheral blood mononuclear cells, DC= dendritic cells, %=percentage

4.4 Results

Patient Characteristics.

Between August 2002 and December 2003, 8 patients with progressive mRCC were included in the study protocol, according to the inclusion criteria. All patients signed informed consent approved by the local ethical committee. Patient nr. 1 dropped out because of the development of pleural effusion and rapid deterioration of the performance status. Patient 6 mentioned visual dysfunction during the visit following the leukapheresis and radiological evaluation revealed cerebral metastasis, which led to the withdrawal of this patient from the study protocol.

DC culture.

Sterile, pyrogene free mature DC were obtained for all patients. FACS analysis revealed a typical mature phenotype of the DC (figure 1). A summary of the amount of PBMC and DC harvested and cultured is presented in table 1.

Toxicity.

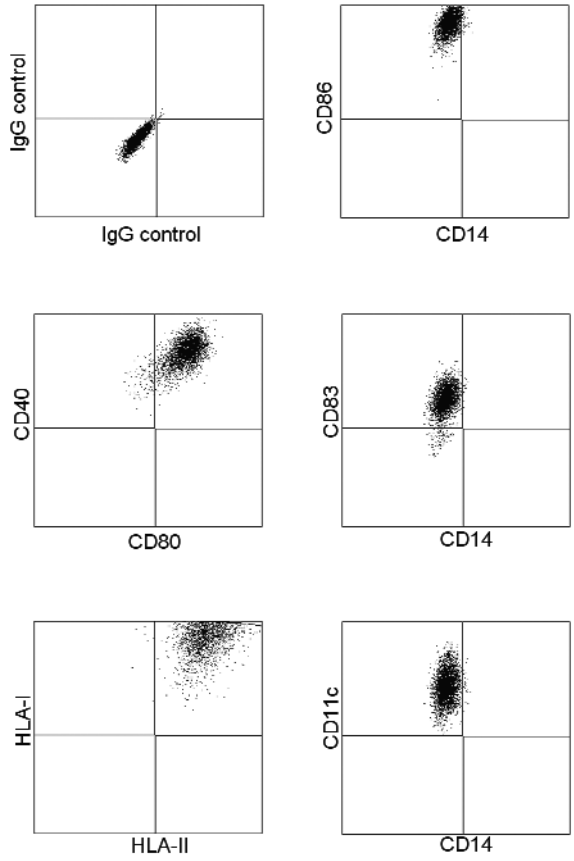
No significant toxicity (above grade II) due to the DC-based vaccines was observed. Some patients experienced flu-like symptoms the day of the vaccination, but no additional treatment or hospitalization was needed. After the second injection local reaction (redness, induration, pruritis) was observed in most patients for approximately 48 hours following the vaccination.

Table 2
Delayed-type-hypersensitivity reactivity and - infiltrating lymphocyteoutgrowth

Pt nr	PBS	KLH	CA9p249	CA9p254	DC	DC-KLH	DC-KLH/ CA9p249	DC-KLH/ CA9p254
2	-	-	-	-	-	+* (6.5)	+* (9.0)	-
3	-	-	+* (4.0)#	-	-	+* (2.5)	+* (2.5)	+* (3.2)
4	-	-	-	-	+* (<0.3)	+* (<0.3)	+* (<0.3)	+* (<0.3)
5	-	-	+* (1.4)	-	-	+	+* (0.6)	+* (2.0)
7	-	-	-	-	-	+* (<0.3)	+* (<0.3)	+* (<0.3)
8	-	-	+* (<0.3)	-	+* (0.5)	+* (1.3)	+* (0.7)	+* (0.6)

A week after the 5th vaccination (week 13), patients received 8 injections to evaluate delayed-type-hypersensitivity reactivity. Patients were injected with PBS, KLH, CA9p254, CA9p249, unpulsed DC, DC pulsed with KLH and DC pulsed with KLH + CA9p254 and KLH + CA9p249 respectively. A DTH injection site was considered positive when an induration of > 0.5cm was observed. -: No DTH reaction, +: positive DTH reaction, *: biopsy of DTH reaction collected, #: numbers between brackets represent outgrowth of DIL x 10^6, harvested after 2-3 weeks culture.

Figure 1
FACS analysis of monocyte-derived mature DC



Clinical outcome.

Evaluation of tumor growth by CT-scanning performed 4 weeks after the 5th vaccination revealed progressive disease in all 6 patients receiving the DC vaccination.

Evaluation of humoral responses.

Serum samples taken at baseline and at week 4, 7, 13 and 17 were analyzed for the presence of antibodies against KLH or CA9-peptide. Antibodies against KLH were absent at baseline. During the trial an increase of anti-KLH antibodies up to week 17 was observed in all patients (figure 2). No anti-CA9 peptide specific antibodies were observed.

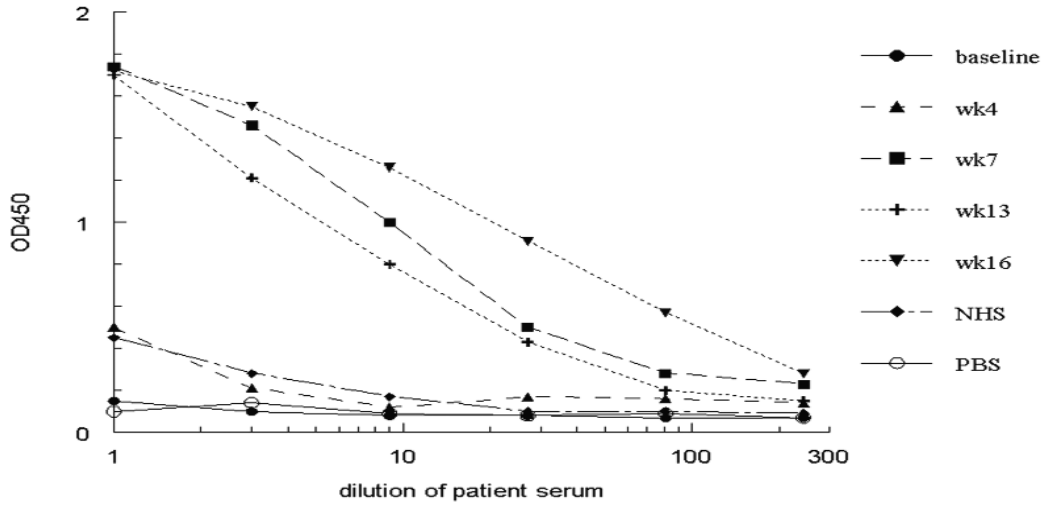
Evaluation of cellular immune responses in peripheral blood.

PBL phenotyping showed no change
in the distribution pattern of CD3+, CD4+, CD8+ and CD56+ cells in time (baseline, week 4, week 13). The percentage DimerX-positive PBL varied from 0.0% to 0.3%. Analysis of the secretion of cytokines into the supernatant of the T-cell stimulation assay of PBL showed a gradual increase in Th2 cytokines (Il5 and IL13) from baseline to week 13 in 4/6 patients (figure 3). For the other cytokines and conditions the results were highly variable without any apparent distinguishable pattern.

Delayed-Type Hypersensitivity (DTH).

No measurable DTH reactivity was observed at baseline. In contrast, DTH was observed in almost all KLH challenges (table 2). Clusters of infiltrating leukocytes were observed in all biopsies collected, which consisted of CD4+ and CD8+ cells at approximate equal numbers. The amount of DTH-infiltrating leukocytes retrieved after 3-4 week culture varied substantially between biopsies and patients (table 2). Phenotypic analysis of DIL showed virtually exclusive outgrowth of CD4+ T-cells in the evaluated patients, regardless of which challenge

Figure 2
Humoral anti-KLH response



KLH-ELISA of sera from a representative patient. The serum of a healthy donor (NHS) was used as negative control.

was studied, including unpulsed DC. The data of a representative patient are shown in table 3. Analysis of cytokines secreted by DIL revealed high cytokine levels of all cytokines examined in the supernatant of days 2 and day 9. A slight bias toward a Th2 cytokine profile was observed (results not shown).

In 2 biopsies sufficient DIL were available to perform a cytotoxicity assay. No efficient peptide-specific lysis of HLA-matched, CA9-positive target cells was observed.

Tetramer staining did not reveal any tetramer-positive cell in any of the skin biopsies.

4.5 Discussion

Dendritic cell (DC) vaccines have demonstrated the possibility to induce antitumor activity in RCC patients [9,10,15]. Appropriate DC maturation and activation are essential components to achieve clinical responses, albeit that only a minority of patients responded even when adequately matured DC were administered. In RCC, vaccination with tumor-lysate pulsed mature DC resulted in clinical responses, and vaccination with mature, autologous DC transfected with total tumor RNA showed vaccine-induced T cell responses against various antigens, including CA9 [10,15]. This antigen is expressed in all clear cell RCC, but not in most normal

Table 3
Phenotypic analysis of DTH-infiltrating leukocytes of patient 5

Origin biopsy	% CD3	% CD4	% CD8	% CD56
Normal skin	65	32	0	4
CA9p249	92	93	2	2
KLH	88	84	2	8
DC-KLH/CA9p249	87	87	3	7
DC-KLH/CA9p254	91	86	1	5

Following approximately 3 weeks of culture, outgrowing DTH-infiltrating leukocytes were evaluated by FACS analysis.

tissues [11,12]. Considering the restricted tissue distribution, the identification of MHC class I and MHC class II CA9-derived peptides with the ability to induce CD8 + CTL and CD4 + DR-restricted Th cells and that vaccination with total tumor RNA loaded DC leads to CA9 specific responses we hypothesized that CA9-peptide vaccination might lead to induction of CA9 specific CTL and antitumor responses. To investigate whether vaccination with CA9-peptide loaded DC could induce CA9-specific responses, a clinical trial in patients with progressive mRCC was performed. As anticipated, and in accordance with comparable clinical trials, no significant side effects were observed. Unfortunately, no change in the disease-course of the RCC patients was noted.

One of the main objectives of this clinical trial was to explore whether CA9 peptide-loaded DC vaccination could induce CA9-specific immune responses. Almost all challenges with pulsed DC demonstrated DTH reactivity, whereas peptide challenges showed very little reactivity: KLH and CA9p254 were negative and in only three cases a positive DTH reaction was noted against CA9p249. The reactivity against the CA9-peptide pulsed DC may not reflect CA9-peptide reactivity, but rather KLH-directed reactivity, since all CA9-peptide pulsed DC were also pulsed with KLH. Because DTH reactivity has not been predictive for a successful response to vaccination and because we were unable to distinguish between KLH and CA9-directed DTH reactivity we examined DTH biopsies. A recent study has suggested that DTH biopsies may be a valuable and reliable source for immunomonitoring purposes [16]. We investigated skin biopsies of DTH sites by immunohistochemistry and analysed leukocytes outgrowing from DTH biopsies. Although immunohistochemical analysis of DTH biopsies revealed CD4+ and CD8+ infiltrating leukocytes, we did not observe outgrowth of CA9-peptide specific cells in any of the biopsies. Outgrowing leukocytes consisted almost exclusively of CD4+ cells. In line with the CD4+ phenotype, analysis of cytokine production showed a slight bias towards a Th2 cytokine profile (Il5 and IL13). Since the outgrowing cells could be a poor representation of the infiltrate due to selection, in situ tetramer staining on the skin biopsies was performed. Unfortunately, in situ-tetramer staining also failed to detect CA9-specific

Figure 3
Evaluation of cytokine production

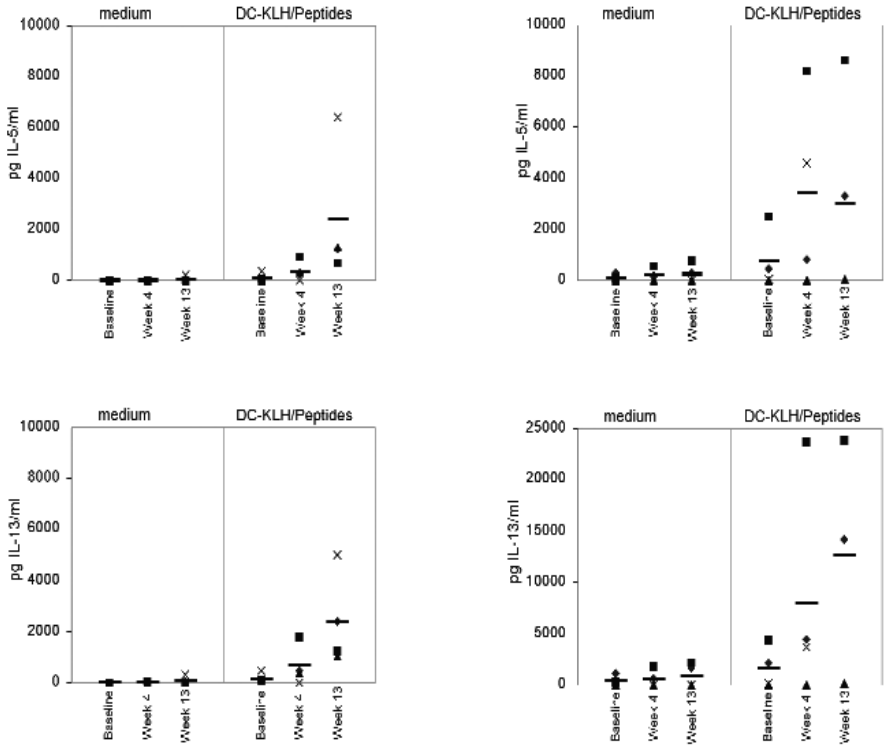


Figure 3

Cytokine secretion of PBL cultured in the absence or presense of peptide-loaded DC. A, B: IL5 secretion 2 days (A) or 9 days (B) after stimulation. C, D: IL13 secretion 2 days (C) or 9 days (D) after stimulation. ▬ =Mean value.

T cells. I.e, no specific CA9-related cellular immune response was observed in any of the DTH. The infiltrating leukocytes observed in the DTH biopsies are most likely the result of the vast amount of locally produced chemokines by the injected mature DC.

Monitoring of humoral responses revealed anti-KLH IgG responses in all patients, but anti-CA9 peptide IgG could not be detected. The anti-KLH IgG response is in agreement with other studies, and confirms the functionality of the DC used.

The absence of anti-CA9 peptide IgG and T cell responses suggests that these peptides are poorly immunogenic in vivo despite convincing in vitro evidence demonstrating their capacity to induce genuine immune responses [13,14]. Alternatively, the complete CA9 protein may not contain immunogenic peptides. However, vaccination of mRCC patients with RCC tumor RNA loaded DC lead to a bona-fide expansion of CA9 specific CTL, demonstrating that the protein does contain immunogenic peptides[16]. Secondly, in a comparable approach, Uemura et al. immunized progressive, cytokine refractory metastatic RCC patients with HLA-A24 restricted peptides emulsified in Montanide (Uemura, pers. commun.). This vaccination resulted in induction of powerful CA9-specific, HLA-restricted lytic activity against RCC cells in

most patients and rapid and high production of anti-CA9 antibody responses. Moreover, clinical responses were observed. The above clearly suggest the possibility to induce CA9 specific immune responses. Future studies should focus on other CA9-related peptides or alternatively, focus on immunization with whole CA9 protein, because this would result in presentation of all possible CA9-peptides, alleviating the need for HLA-matched vaccination.

As mentioned, new treatment modalities (Bevacizumab, Sutent (SU11248), Sorafenib (BAY 43-9006)) that focus on gene products upregulated as a consequence of mutations in the VHL-gene show promising results [3-6]. Of interest, there is a clear link between CA9 and VHL. It will be interesting to combine various treatments: e.g. reduction of VEGF-induced immunosuppression by bevacizumab to improve specific anti-tumor immune responses induced by vaccination strategies. Secondly, the extend of clinical cross-resistance is unknown, which provides a rational for combination.

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CHAPTER 5

A PHASE II TRIAL OF CHIMERIC MONOCLONAL ANTIBODY G250 FOR ADVANCED RENAL CELL CARCINOMA PATIENTS.

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5.1 Abstract

Purpose: Chimeric monoclonal antibody G250 (WX-G250) binds to a cell surface antigen found on >85% of renal cell carcinoma (RCC). A multicenter phase II study was performed to evaluate the safety and efficacy of WX-G250 in metastatic RCC (mRCC) patients.

Patients & Methods: Thirty-six patients with mRCC were included. WX-G250 was given weekly by IV infusion for 12 weeks. Patients with stable disease or response were eligible to receive additional treatment for 8 weeks.

Results: None of the 36 enrolled patients experienced any drug related grade III or IV toxicity. Only 3 patients had grade II toxicity possibly related to the study medication. Ten patients obtained a stable disease and received extended treatment. One complete response and a significant regression was observed during the follow-up of the treatment. Five patients with progressive disease at study entry were stable for more than 6 months after study entry. The median survival after treatment start was 15 months.

Conclusion: The weekly schedule of WX-G250 was well tolerated. With a median survival of 15 months after start of this treatment and two late clinical responses, WX-G250 seems to be able to modulate mRCC. To improve activity of WX-G250 specific antibody dependent cellular cytotoxicity (ADCC) and the clinical response rate, currently combinations of WX-G250 with cytokines are in phase-II trials.

5.2 Introduction

Patients with metastasized renal cell carcinoma (mRCC) have a poor prognosis. Up to one third of the newly diagnosed patients present with metastatic disease and another 33% of the patients develop recurrent disease after surgery with curative intent [1]. In view of well documented spontaneous remissions, the immune system appears to play a role in the natural history of the disease [2]. Against this background the main focus of treatment of mRCC has been on immunotherapy. Currently, interferon-alpha (IFN-a) and interleukine-2 (IL-2) are most commonly used in the treatment of mRCC, either alone or in combination [3,4]. However, the response rates are low and the toxicity significant. Therefore, new treatment modalities are being studied [5,6].

Currently, several monoclonal antibodies (mAb) have been approved for the use of cancer treatment, e.g. in non Hodgkin's lymphoma [7]. In RCC, the chimeric monoclonal antibody WX-G250 has been identified and developed for both diagnostic and therapeutic purposes [8,9]. It recognizes the Carbonic Anhydrase-IX$^{G250/MN}$ (CA9) antigen expressed in >95% of RCC of the clear cell type [10]. Moreover, CA9 is not expressed in normal kidney tissue and in other normal tissues the expression is highly restricted and limited to large bile ducts and gastric epithelium [8]. The observation that WX-G250 was able to mediate antibody dependent cellular cytotoxicity (ADCC) against several RCC cell lines [11] led to the initiation of the present study.

5.3 Patient & Methods

Study Design

A phase II, prospective, open-label, single-arm, multicenter study was initiated. Patients with mRCC received weekly doses of 50 mg WX-G250 for 12 weeks. Study objectives included the evaluation of efficacy, safety, immunogenicity and biological activity of the WX-G250. Patients were treated at five medical centers: Johannes-Gutenberg University Hospital Mainz, Germany (enrolled patients: n=14); Hospital Northwest Frankfurt/Main, Germany (n=11); University Medical Center Nijmegen, The Netherlands (n=6); Philipps University Hospital, Marburg, Germany (n=4); and Daniel den Hoed Cancer Center, Erasmus MC, Rotterdam, The Netherlands (n=1). All patients signed an informed consent approved by the local ethical committees of the respective hospitals. Patients were recruited between June 14, 2000 and December 20, 2000.

Patient population:

All patients had primary RCC of clear cell histology and prior nephrectomy. Inclusion criteria were: bidimensionally measurable disease with lesions < 5 cm in diameter and at least one lesion of > 1 cm; life expectancy > 28 weeks; Karnofsky Performance status > 70%; seronegative for human immuno-deficiency virus (HIV) and hepatitis B surface antigen; absolute neutrophil count (ANC) 2.0 x 10^8/dL; platelet count 100 x 10^8/dL; hemoglobin > 6.5 mmol/L (equals 10.5 g/dL); total bilirubin < 1.5 x upper limit of normal (ULN); AST, ALT <3 x ULN; (<5 x ULN if liver metastases present). Patients with clinical signs of CNS metastases and patients with bone metastases only were excluded.

Antibody:

WX-G250 is a human/mouse chimeric mAb derived from murine mAb G250 by recombinant DNA techniques. WX-G250 is similar regarding binding affinity and specificity in comparison with the murine G250 IgG [9]. All clinical lots were generated starting from a cell bank according to current GMP requirements. The antibody was supplied by Wilex AG (München, Germany).

Treatment:

Patients received 50 mg WX-G250 once a week by intravenous infusion for 12 weeks followed by radiographic evaluation 4 weeks later. Patients with stable disease or a tumor response were eligible for extended treatment, consisting of 8 additional weekly infusions of WX-G250. The WX-G250 was dissolved in 10 ml solution for injection, pre-filtered through a low-protein binding 0.2um filter and infused in 50-100 ml normal saline over a period of 30 minutes. Patients were observed for at least two hours for blood pressure, pulse, temperature and allergic reactions after completion of the infusion. The 50 mg dose was chosen based on the following observations: 1) a single dose of 50 mg WX-G250 (radiolabeled with ^{131}Iodine) was safe in 16 RCC patients [9], 2) 50 mg WX-G250 was sufficient to reach maximum uptake of the antibody in RCC [9] and 3) the results of a multidose phase I study with escalating WX-G250 doses showed that weekly doses of 25 mg/m^2 (corresponding to the 50 mg dose used in this study) caused no clinical or laboratory drug related adverse events during 6 weeks treatment [12]. The interval of the application was selected based on the WX-G250 serum half-life of approximately 70 hours [9]. Therefore it was expected that levels of WX-G250 adequate to induce ADCC would persist for at least one week [11]. Weekly administration was not expected to lead to significant cumulative increase of serum levels of WX-G250.

Table 1
Charactistics of the evaluated patients

Pt no.	*Sex*	*Age*	*Prior tumor therapy (best response)*	*Karnofski*	*Clinical status at start*	*Metastasis*	*Number of infusions*	*Response after first cycle*	*Survival (months; Until January 2002)*
1	M	68	IFN+Vinbl (PD)	90-100%	PD	Adrenal	12	PD	6
2	F	45	IFN+Vinbl (PD)	70%	PD	Lung & liver	4	PD	2
3	F	75	None	80%	PD	Lung	12	PD	4
4	M	51	None	80%	PD	Lung, liver & LN	11	PD	3
5	F	77	None	90%	PD	Lung	5	PD	2
6*	M	66	IFN+Vinbl (SD) IFN+Vinbl (PD) 5FU+CF (PD)	80%	PD	Lung & liver	20	SD	18+
7	F	58	Radiation (PD)	90-100%	PD	Lung	12	PD	18+
8	M	77	IFN+Vinbl 5FU+CF	80%	PD	Lung & bone	4	PD	Date unknown
9*	M	68	IFN+Vinbl (SD) 5FU+CF (SD)	90-100%	PD	Lung, pleura, adrenal	20	SD	18+
10	M	69	IL-2/IFN/5FU (SD) IL-2/IFN/5FU red (PD)	90-100%	PD	Lung & liver	12	PD	18+
11	M	66	None	90-100%	PD	Lung & LN	12	PD	17
12	F	67	None	90-100%	UN	Liver, spleen & LN	12	PD	18+
13	F	64	IFN+Vinbl (PD)	90-100%	PD	Lung & liver	11	PD	4
14	M	72	None	90-100%	PD	Lung	7	PD	1
15	M	51	IFN+Vinbl (PD)	90-100%	PD	Lung, LN, pancreas	12	PD	17+
16	M	54	IFN+Vinbl (PD) IFN+cis RA (PD) 5FU+CF (PD)	90-100%	PD	LN, contralateral kidney & psoas muscle	12	SD	17+
17	M	67	IFN+Vinbl	80%	PD	Lung	5	PD	7
18	M	50	IFN (PD)	90-100%	PD	Lung	12	PD	9
19	M	43	None	90-100%	PD	Lung & LN	12	PD	8
20	F	60	None	80%	UN	LN	12	PD	11
21*	M	69	None	90-100%	UN	lung & LN	20	SD	16+
22*	M	70	None	90-100%	UN	LN	20	SD	13
23*	M	61	None	80%	PD	Lung, LN, & Liver	20	SD	16+
24*	M	56	IL-2 (SD) DC vaccination (SD)	90-100%	PD	LN	20	SD	15+
25*	F	52	IFN+cis RA (CR)	90-100%	PD	Other kidney	20	SD	15+
26	M	69	IL-2/IFN/5FU (PD)	80%	PD	Lung & LN	12	PD	15+
27*	M	57	IL-2/IFN/5FU (PR)	90-100%	UN	Lung	20	SD	15+
28*	F	76	None	90-100%	PD	Lung & adrenal	20	SD	14
29	M	66	IL-2/IFN/5FU (SD) IL-2 inhalation (PD)	80%	PD	Lung & LN	12	PD	15+
30	F	65	IL-2/IFN/5FU (PD) Toremifene + Vinbl (PD)	90-100%	PD	Lung & LN	12	PD	14+
31	F	70	IFN+Vinbl (PR) IFN+Vinbl (PD) IFN+Vinbl (PD)	90-100%	PD	Lung	12	PD	14+
32	M	76	None	90-100%	PD	Lung	12	PD	14+
33	M	71	None	80%	PD	LN & Pleura	12	PD	14+
34	M	57	Vaccination (PD)	90-100%	PD	Lung	12	PD	7
35	M	67	IFN+cis RA (SD)	90-100%	PD	Lung & LN	10	PD	5
36*	F	73	None	80%	PD	Lung	20	SD	13+

*: Patients who received extended treatment.
Abbreviations: Sex: M= male, F= female. Response: PD= progressive disease, SD= stable disease, PR= partial response, CR= complete response, UN= unknown. Metastasis: LN= lymphnode. Prior treatment: IFN= interferon-alpha, Vinbl= vinblastine, 5FU= 5-fluorouracil, CF= calcium folinate, IL-2= interleukin-2, cis RA= cis-retinoic acid, DC= dendritic cell.

HACA evaluation:

A sandwich type ELISA was used to analyze human antibody chimeric antibody (HACA) levels in serum of patients as described previously [9]. In brief, unconjugated WX-G250 was coated onto ELISA plates, followed by incubation with the serum of the patient. Detection of anti WX-G250 HACA was performed using biotinylated WX-G250, and a streptavidin-biotinylated peroxidase complex. In 22 patients, pretreatment HACA levels were determined. Blood samples for HACA evaluation were taken before each infusion with WX-G250 and regularly during follow-up. The serum of a patient with positive HACA levels obtained from an unrelated WX-G250 trial was used as positive control. The calibration curve for the quantification of anti WX-G250 HACA was generated using the WX-G250 anti-idiotypic antibody NUH82 [13]. The limit of detection (LOD) of this ELISA was 8.3 ng/ml (NUH82); limit of quantification (LOQ) was 27 ng/ml.

Biological activity, ADCC:

At baseline and prior to WX-G250 infusion at week 4 and week 12, venous blood samples were collected in 4 sodium heparin vacutainer^{TM} CPT^{TM} tubes (Becton, Dickinson & company, The Netherlands). The tubes were centrifuged (1500g, 30 minutes) at the treatment site and shipped overnight to the evaluator. Peripheral blood mononuclear cells (PBMC) were collected and frozen in 50%FCS / 10%DMSO medium and stored at -170 0C until the time of evaluation. To confirm the presence and proportion of natural killer (NK) lymphocytes, immunophenotyping was performed. The biological activity of WX-G250 was analyzed by 4-hour 51chromium release assays at different effector to target cell ratios using autologous cryopreserved PBMC as effector cells. Target cells were SKRC-17pMW1-cl4 (CA9 antigen transfected RCC cell line) and SKRC-17 (CA9 antigen negative RCC cell line). As controls for lytic activity of the PBMC, P815 cells were used as target cells in the presence of anti-P815 rabbit antiserum ("classic"-ADCC). To evaluate non-specific cellular lytic activity, K562 and Daudi cells were used as additional target cells, in the absence of WX-G250 antibody. The cytolytic activities were expressed as percentage weighted mean of specific cytolysis (WMSL) [14]. ADCC measured against the target cells incubated with the WX-G250 antibody (1 mg/ml) was corrected for the ADCC seen against the target cells incubated without the WX-G250 antibody.

Patient monitoring and efficacy evaluation:

Patients were monitored for safety, biological activity of WX-G250, HACA development and clinical antitumor effects. At baseline and weeks 4, 12 and 16 medical histories, physical exa-

Table 2
Adverse events registered during the study, stratified by NCI class and Grade.

NCI class	*Events (N)*	*Pts (N)*	*Pts (%)*	*n.a.*	*Gr I*	*Gr II*	*Gr III*	*Gr IV*
Pain	32	15	41.7	0	12	13	7	0
Gastrointestinal	26	14	38.9	0	14	12	0	0
Pulmonary	20	9	25.0	0	5	5	6	4
Cardiovasculair (General)	17	12	33.3	0	8	4	5	0
Constitutional Symtoms	15	9	25.0	0	10	4	1	0
Neurology	11	8	22.2	0	3	6	2	0
Blood/ Bone marrow	10	5	13.9	0	1	5	4	0
Not to classify	7	5	13.9	4	3	0	0	0
Renal/ Genitourinary	5	4	11.1	0	2	1	2	0
Hemorrhage	5	3	8.3	0	3	0	1	1
Hepatic	4	2	5.6	0	0	0	4	0
Infection/ Febrile Neutropenia	3	3	8.3	1	1	1	0	0
Metabolic/ Laboratory	3	3	8.3	0	1	1	1	0
Allergy/ Immunology	1	1	2.8	0	0	1	0	0
Ocular/ Visual	1	1	2.8	0	0	1	0	0
Total	160	30	83.3	5	63	54	33	5

Abrevations: Pts= Patients, Gr= Grade, n.a.= Grade not specified.

minations, urinalysis, and laboratory studies including complete blood count and chemistry panel were performed. Toxicity was evaluated according to the Common Toxicity Criteria (Version 2.0, Apr 1999, National Cancer Institute). Computed tomography scans of the thorax and abdomen were performed at baseline, at week 16 and 4 weeks after the extended treatment, when applicable. Tumor responses were evaluated according to the following WHO criteria: Complete response (CR): The disappearance of all known disease determined by two evaluations not less than four weeks apart. Partial response (PR): >50% decrease in the sum of products of largest and perpendicular diameters of the lesions that have been measured to determine the effect of therapy by two evaluations not less than four weeks apart, in the absence of new lesions or progression of any lesion. Stable disease (SD): The total tumor size has less than 50% and the increase is less than 25% in the size of one or more measurable lesions. Progressing disease (PD): a 25% or more increase in the size of one or more measurable lesions, or the appearance of new lesions. For the CT and MRI evaluations an independent central review was performed as requested by protocol.

Statistical methods:

The study had a two-stage design. In the first stage, 32 evaluable patients with mRCC were included. Twenty-two additional patients were allowed to participate (54 patients in total) if at least three objective responses were observed at the time of the radiological evaluation at week 16. The study had to be terminated if less than three patients or more than five had an objective response. At the maximum enrollment number of 54 patients the trial was powered at 80%

Table 3
Immunophenotyping of ADCC

Subject	*Treatment day*	*Immunophenotyping % NK cells*	*Cytolytic avtivity cG250-ADCC*	*Cytolytic avtivity "classic" ADCC*	*NK-activity*
#01	0, 21, 76	5, 4, 4	16, 9, 10	40, 29, 27	-, 5, 4
#06	0, 21, 77	9, 9, 7	14, 14, 11	38, 32, 40	16, 19, -
#07	0, 21, 77	9, 11, 9	8, 16, 5	94, 100, 50	-, 35, -
#09	0, 28, 77	7, 8, 11	1, 1, 2	45, 72, 49	-, 24, 28
#10	0, 21, 77	6, 5, 2	0, 1, 0	40, 32, 38	-, -, -
#11	0, 21, 77	8, 8, 5	10, 7, 5	55, 42, 48	-, -, -
#12	0, 21, 77	ND	3, 3, 2	32, 39, 36	-, -, -
#13	0, 21, 78	ND	2, 2, 2	27, 27, 22	-, -, -
#15	0, 21, 77	ND	3, 1, 1	20, 16, 21	-, -, -
#16	0, 21, 78	14, 13, 10	14, 25, 26	37, 47, 50	4, 8, 6
#18	0, 29, 85	1, 1, 1	0, 2, 0	49, 37, 15	0, 1, 0
#19	0, 21, 77	6, 2, 1	1, 0, 0	34, 38, 9	0, 0, 0
#20	0, 21, 77	1, 1, 2	1, 2, 3	2, 6, 8	0, 2, 1
#21	0, 21, 77	12, 12, 7	4, 4, 3	25, 23, 17	5, 5, 3
#22	-7, 21, 77	7, 4, 5	15, 7, -	31, 40, -	29, 16, -
#24	-8, 21, 77	9, 4, 4	6, 5, 5	55, 36, 41	17, 16, 45
#25	0, 21, 77	7, 7, 5	14, 15, 18	32, 37, 36	24, 23, 38
#26	0, 50, 78	6, 4, 13	0, 0, 3	16, 20, 40	3, 6, 20
#27	0, 21, 77	5, 4, 5	7, 9, 11	44, 32, 32	-, -, -
#28	0, 28, 84	2, 3, 3	0, 0, 0	24, 22, 19	4, 4, 4
#29	0, 28, 78	1, 1, 2	0, 1, 2	9, 8, 17	0, 0, 1

Abrevations: NK= natural killer cell, ADCC= antibody dependent cellular cytotoxicity, ND= No data. %NK cells = percentage NK cells of viable leukocytes. Cytolytic activities expressed as %WMSL at Effector-to-Target-ratio of 20:1.cG250-ADCC: cG250 mAb induced cytolysis of G250-ligand expressing target cell. 'classic'-ADCC: anti-P815 Ab induced cytolysis of P815 target cell (=positive control). NK-activity: cytolysis of NK-sensitive target cell K562.

and based on a < 0.05 to detect a difference between an assumed spontaneous response rate of 5% and an underlying true response rate of 15%.

5.4 Results

Patient characteristics (table 1)

From June 2000 to December 2000 a total of 36 RCC patients were included. The study population (mean age 64 years, range: 42-77 years, 12 females and 24 male) displayed a total of 107 target lesions in different sites at study entry. In 29 patients (80.5%) disease progression was documented before study entry, for 4 patients the status was not rated, the remaining 3 were not progressive. Of the 36 evaluated patients, 20 patients received prior systemic treatment that resulted in one CR, one PR, 3 SD. Fifteen patients did not respond to cytokine treatment.

Safety evaluation (table 2)

All 36 patients received at least one dose of WX-G250, and were assessed for safety. No dose reduction was necessary. Of the recruited 36 patients, 30 experienced a total of 160 adverse events during the course of the study. All grade 3 and 4 toxicities were considered not to be related to the study medication. An overview of the observed toxicities is given in table 2. The grade 4 toxicities consisted of dyspnea and pulmonary insufficiency (patient 4), dyspnea at rest (3), respiratory insufficiency (5) and gastrointestinal bleeding (13). Two patients (4, 5) died during the study (defined as death within 30 days after the last application of study medication), both due to progression of their disease. None of the serious adverse events (including the deaths) were related to study medication, but due to underlying disease.

Evaluation of HACA:

In the evaluated pre-treatment sera of 22 patients, no HACA levels were detected. In three samples of two patients (18, 27) levels higher than the LOD could reproducibly be detected. Serum of patient 18 showed an equivalent of 76 ng/ml anti-G250 antibody NUH82 at treatment week 12. In sera of patient 27 an equivalent of 49 ng/ml anti-G250 antibody NUH82 at extension week 1, and a value between the LOD and LOQ at extension week 8 could be detected.

Immunological monitoring (table 3):

Of eighteen patients phenotypic analysis of PBMC was performed (table 3). Consecutive samples of every patient were tested simultaneously to exclude assay-to-assay variation. In 15 of these 18 patients the percentage of NK cells was >2% of leukocytes in at least one of the samples (range: 1% to 14%). The percentage NK cells in individual patients remained rather constant during treatment. Consecutive samples of twenty-one patients were tested for ADCC capacity (table 3). Nine out of 21 patients showed moderate (5-25%) to high (>25%) levels of WX-G250-ADCC activity. Five out of 21 patients showed low levels of WX-G250-ADCC and 7 out of 21 patients showed no WX-G250-ADCC activity. Nineteen of 21 patients tested showed normal levels of "classic"-ADCC activity. In general, WX-G250 related ADCC remained unchanged during the treatment.

Clinical response (table 1):

Clinical progression before week 16 was seen in 8 patients. In 4 of these patients progression occurred within 6 weeks after study entry and for those patients an additional patient was included, in accordance with the protocol. Patient 16 was judged as SD according to an outside radiologist, but was subsequently lost to follow-up. Independent central tumor assessment was performed at week 16 in the remaining 27 patients. Radiographically proven progression of the disease was observed in seventeen patients, who therefore did not qualify for extended treatment with WX-G250. Ten patients showed stable disease after 12 weeks of treatment and continued treatment consisting of eight additional weekly infusions of WX-G250. The clinical status of these patients was assessed in week 24 and eight out of ten patients still showed stable disease. After the end of extension treatment the patients were routinely followed at three monthly intervals. The follow-up evaluation revealed a minor response in patient 9 in week 44 and a complete response in patient 21 in week 38. Evaluation of survival showed a median time to death of 15 months after the start of the WX-G250 treatment. The evaluation of the first stage of the study did not show 3 objective responses at the time of radiological evaluation at week 16. Consequently, the study did not enter the second stage of the trial.

5.5 Discussion

We performed a phase II, prospective, open-label, single-arm, multicenter study in patients with advanced RCC receiving weekly doses of 50mg WX-G250 for 12 weeks to evaluate the potential therapeutic effect of WX-G250. WX-G250 recognizes the antigen CA9 that is homogeneously expressed in virtually all clear cell RCC. More importantly, expression in normal tissue is extremely limited, making this antigen an attractive target for immunotherapy.

During the trial no serious drug related clinical or laboratory adverse events were observed, and no dose adjustment was necessary. Therefore the toxicity profile of WX-G250 is favorable and comparable with prior studies evaluating WX-G250 [9]. WX-G250 is a murine-human chimeric antibody; and may consequently induce the development of HACA. In the current trial low levels of HACA were detected in only two out of 35 patients (8 and 27). The HACA levels were not associated with clinical symptoms, which is in accordance with earlier studies evaluating WX-G250 [9]. This study demonstrates that multiple infusions with WX-G250 are well tolerated and can be given safely.

Evaluation of the first stage of the trial showed less than 3 objective responses needed to continue into the second stage of the trial and thus the trial was terminated. Nevertheless, follow-

up revealed two objective responses: Patient 9 presented at study entry with multiple progressive metastases. After both the first course and extended treatment, the lesions were rated as stable. In the following months, radiological evaluation showed a decrease of the target lesions. Taking all measurable lesions into account, the response remained slightly below the 50% decrease required for a partial remission, making it a minor response. Patient 21 started treatment with WX-G250 immediately after nephrectomy. At study entry he presented with lymph node metastases and multiple pulmonary lesions. The size of the lesions decreased 29% between week 16 and week 24, and was therefore rated as stable disease. In week 38 after study start a complete remission of all the lesions was documented. Since spontaneous remissions of RCC metastases after removal of the primary tumor do occur [2], we cannot exclude that the clinical observation of patient 21 was a reflection of the natural history of the disease, unrelated to WX-G250.

Eleven patients were rated with stable disease at the radiological evaluation at week 16. Of those patients, 8 showed progressive disease at study entry, of which 2 patients (6, 23) with previously progressive liver lesions and others after progression under previous immunotherapy regimens. These are events that rarely occur, even after "standard" cytokine treatment [15-17]. Collectively, these results suggest that WX-G250 has the capacity of modulating the natural history of metastasized RCC with a safe toxicity profile.

ADCC is suggested to be the main effector mechanism of WX-G250 [11] and is mediated by the interaction between the Fc-region of an antibody bound to a tumor cell and the Fcg receptors on immune effector cells, such as neutrophils, macrophages and natural killer (NK) cells [18]. In our trial, the levels of WX-G250 mediated ADCC differed between the patients: 42% of the patients showed moderate ADCC, whereas 33% showed no ADCC at all. There was no clear correlation between the proportion of NK-cells and the level of WX-G250 mediated ADCC and no correlation between the in-vitro levels cytotoxicity and the clinical responses. This high variability in observed ADCC capacity and number of NK-cells was also found in healthy donors (personal observations), suggesting that the observed variation was not the result of the disease status of the patients. Molecular studies have shown significant polymorphism in the genes for the different Fc receptors [19]. These polymorphisms may have important functional consequences. The pattern of Fc gamma RIIIA expression polymorphism is probably correlated with the ability of NK cells to perform ADCC. This may be the reason that no correlation between the proportion of NK-cells and level of WX-G250 mediated ADCC was observed.

In summary, the weekly schedule of intravenous WX-G250 in patients with mRCC was safe and well tolerated. Evaluation of the immunogenecity of WX-G250 demonstrated that an

increased level of HACA does not lead to clinical symptoms. In our trial, 1 complete responder, one minor response and a substantial number of durable disease stabilizations were observed with WX-G250 monotherapy. The median survival after study entry was 15 months. This suggests the capacity of WX-G250 to modulate the natural history of metastatic RCC. Recently was shown that a variety of cytokines e.g. interleukin-2 and interferon-gamma, led to up-regulation of WX-G250 mediated ADCC [20]. Subsequently, phase-II trials optimizing treatment schedules with WX-G250 by combination with cytokines have been initiated.

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CHAPTER 6

A CLINICAL TRIAL WITH CHIMERIC MONOCLONAL ANTIBODY WX-G250 AND LOW-DOSE INTERLEUKIN-2 PULSING SCHEME FOR ADVANCED RENAL CELL CARCINOMA PATIENTS.

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6.1 Abstract

Purpose: WX-G250 is a chimeric monoclonal antibody that binds to Carbonic Anhydrase-IX$^{G250/MN}$, present on >95% of renal cell carcinoma (RCC) of the clear cell subtype. The suggested working mechanism of WX-G250 is by antibody dependent cellular cytotoxicity (ADCC). Because the number of activated ADCC-effector cells can be increased by a low-dose interleukin-2 pulsing schedule (LD-IL-2), a multicenter study was initiated to investigate whether WX-G250 combined with LD-IL-2 can lead to improved clinical outcome of patients with progressive RCC.

Patients and Methods: 35 progressive RCC patients with clear cell RCC received weekly infusions of WX-G250 for 11 weeks combined with a daily LD-IL-2 regimen. Patients were monitored longitudinally for ADCC capacity. Radiological assessment of the metastatic lesions was performed at week 16 and regularly until disease progression.

Results: A durable clinical benefit was achieved in 8 of 35 patients (23%; 3xPR, 5x stabilization >24 weeks) and mean survival was 22 months. In general the treatment was well tolerated with little toxicity. The number of effector cells increased during treatment, but the lytic capacity per cell did not increase. ADCC levels and clinical outcome did not appear to correlate.

Conclusions: WX-G250 in combination with LD-IL-2 in patients with mRCC is safe and well tolerated. With a substantial clinical benefit and a median survival of 22 months in mRCC patients with progressive disease at study entry the combination therapy showed an increased overall survival compared to WX-G250 monotherapy and the survival was at least similar to currently used cytokine regimens, but with a favorable toxicity profile.

6.2 Introduction

The current prognosis of patients with metastasized renal cell carcinoma (mRCC) is poor [1]. Once metastasized, the disease is difficult to treat since mRCC is unresponsive to conventional treatment, and, although non-specific immunotherapy has convincingly shown its ability to induce long-term clinical responses in a subset of patients [2], the overall response rate is low and side-effects are significant [3]. Consequently, the main focus for new treatments in mRCC concerns antiangiogenic therapy (e.g. SU11248, BAY- 43-9006 (BAY), and bevacizumab [4-6]) as well as immunotherapeutic modalities with more specificity against RCC and less side effects [3]. Currently, several monoclonal antibodies (mAb) have become standard treatment for selected malignancies, with remarkable clinical results and a very favorable toxicity profile [7,8]. In RCC, the antibody WX-G250 has been identified [9,10]. It recognizes the Carbonic Anhydrase-IX$^{G250/MN}$ (CA9) antigen that is expressed in >95% of RCC of the clear cell type [11]. Moreover, no expression can be detected in most normal tissues, including the normal kidney [9]. Clinical studies with radiolabeled WX-G250 have revealed excellent targeting to tumor lesions, corroborating the CA9 antigen expression pattern in metastatic lesions and potential use of WX-G250 as therapeutic modality [12]. Monotherapy with WX-G250 in 36 patients with mRCC demonstrated one objective responder, one minor response, and a median survival of 16 months, suggesting that WX-G250 might have anti-tumor activity [13]. In-vitro studies have demonstrated that WX-G250 can elicit antibody dependent cellular cytotoxicity (ADCC) [14] which can be upregulated by IL-2 [15,16]. We hypothesized that WX-G250 combined with a single daily subcutaneous injection low dose IL-2 (LD-IL-2) could evolve to a new treatment for mRCC.

6.3 Patients and Methods

Study Design & Patient population:

A prospective, open-label, single-arm, multicenter study was initiated. All patients had clear cell mRCC with objective progression at study enrollment according the WHO criteria and prior nephrectomy. Eligible patients had bidimensionally measurable disease with lesions between 1 and 5 cm in diameter and a Karnofsky Performance status >80%. Patients with clinical signs of CNS metastases and patients with bone metastases only were excluded.

Study objectives included the evaluation of safety, induction of human-antibody-chimeric-antibody (HACA), biological activity (ADCC) and the clinical efficacy of WX-G250 in com-

bination with recombinant human IL-2 (rHu-IL-2; ProleukinR; aldesleukin; Chiron Corporation, Emeryville, CA, USA). Patients were treated at Johannes-Gutenberg University Hospital Mainz, Germany and University Medical Center Nijmegen, The Netherlands. All patients signed an informed consent approved by the local ethical committees of the respective hospitals.

The study had a two-stage design. The first 15 patients received WX-G250 and IL-2 according to protocol. If at least 1 objective response (PR or CR) occurred, 15 additional patients were enrolled. Additional patients were included for patients who did not complete the first 6 weeks of treatment.

WX-G250 is a human/mouse chimeric mAb derived from murine mAb G250 by recombinant DNA techniques. All clinical lots were generated starting from a chimeric cell bank according to current GMP requirements. Wilex AG (Munich, Germany) supplied the antibody.

Treatment schedule:

In the first week, IL-2 was administered without WX-G250, to separately observe potential IL-2 side effects. When IL-2 toxicity was judged acceptable, patients received a fixed dose of 20 mg WX-G250 once a week by intravenous infusion for 11 consecutive weeks. Patients received or self-administered the LD-IL-2 for 12 consecutive weeks (Table 1). Patients received 1.8 MIU/day with exception of days 1-3 of weeks 3,5,7,9,11 where 5.4 MIU/day was administered (IL-2-pulsing).

Patients with disease stabilization or tumor shrinkage at week 16 were eligible for extended treatment, consisting of 6 additional infusions of WX-G250 in combination with LD-IL-2 treatment.

HACA evaluation:

A sandwich type ELISA was used to analyze HACA levels in the sera of 28 patients as described previously [10]. WX-G250 was coated onto ELISA plates, followed by incubation with the serum of the patient. Detection of anti WX-G250 HACA was performed using biotinylated WX-G250, and a streptavidin-biotinylated peroxidase complex. Wilex performed all HACA evaluations.

Biological activity, ADCC:

At baseline and prior to WX-G250 infusion at week 2, 6, 10, 12 and week 16, venous blood samples were collected and shipped to the evaluator. Peripheral blood mononuclear cells (PBMC) were collected, frozen in RPMI 1640/ 10%DMSO/ 25% Fetal Calf Serum and stored

Table 1
Treatment schedule

	WX-G250 weekly i.v.	*IL-2 daily s.c.*
Week 1	None	Day 1-7: 1.8 MIU (low dose)
Week 2, 4, 6, 8, 10, 12	20 mg	Day 1-7: 1.8 MIU
Week 3, 5, 7, 9, 11	20 mg	Day 1-3: 5.4 MIU (pulse)
		Day 4-7: 1.8 MIU

Abbreviations: mg= milligrams; MIU= million international units; i.v.= intravenous'; s.c.=subvutaneous; IL-2= interleukin-2.

at -700C. Samples of individual patients were analyzed simultaneously to prevent inter-assay variation.

The cells were thawed and immunophenotyped by flow cytometric analysis (EPICS XL, Beckman-Coulter), for CD3, CD14, CD16, CD45, and CD56. CD56dim and CD56bright cell subpopulations were identified through visual interpretation of the flow cytometric analysis.

ADCC was analyzed by 51chromium release assays as described previously [13]. Cells were cultured overnight in RPMI 1640/10% Human AB serum, diluted to effector:target ratios of 80, 40, 20, and 10: 1 respectively and WX-G250 were added (1 μg/ml). Target cells (SK-RC-52 (CA9pos), SK-RC-17cl4 (CA9pos, transfectant cell line), SK-RC-17cl1 (CA9neg), K562, Daudi) were harvested and labeled with 51Cr for 60 - 90 minutes. Cells were washed and added to PBMC/ WX-G250 and incubated at 37°C for 4-5 hours. Released 51Cr was counted in a liquid scintillation counter (Wallac/LKB). Assays were performed in triplicate. Spontaneous and maximum release was determined by omission of effector cells and addition of 2% Triton X-100 respectively. Specific 51Cr release was calculated as:

$$\frac{\text{Cpm } ^{51}\text{Cr released - Cpm spontaneous } ^{51}\text{Cr released x 100\%}}{\text{Cpm } ^{51}\text{Cr maximum released - Cpm spontaneous } ^{51}\text{Cr released}}$$

Lytic units (LU) were calculated by linear regression analysis for the E:T ratio at which 20% specific lysis was obtained and expressed as LU/ 10E7 effector cells.

Patient monitoring and efficacy evaluation:

At baseline and weeks 2, 6, 10, 12 and 16 medical histories, physical examinations, urinalysis, and laboratory studies including complete blood count and chemistry panel were performed. Toxicity was evaluated according to the Common Toxicity Criteria (Version 2.0, Apr 1999, National Cancer Institute). CT of the thorax and abdomen were performed at baseline, week 16 and 4 weeks after the extended treatment, and at subsequent visits when applicable. Tumor responses were evaluated according to WHO criteria. CT and MRI evaluations were

Table 2
Characteristics of evaluated patients

Pt nr	*Age (Sex)*	*Prior tumor therapy (Best response)*	*Lag between tumornephrectomy and study entry*	*Number of metastatic lesions (location)*	*Clinical response at 16 / 22 weeks*	*Survival in weeks*
1	55 (m)	IL-2/IFN/5FU (SD); IFN/Vinbl/5FU (PD)	> 1 year	2 (lung, LN)	**SD / SD**	104+
2	49 (m)	None	> 1 year	1 (lung)	**SD / SD**	156+
3	73 (m)	None	< 1 year	1 (LN)	PD / NA	35+ §
4	58 (m)	None	< 1 year	1 (lung)	PD / NA	133+
5	55 (m)	Vaccination (PD)	< 1 year	2 (lung, LN)	**SD / SD**	105+
6	49 (m)	None	> 1 year	1 (lung)	PD / NA	33 §
7	51 (m)	None	> 1 year	2 (LN, kidney)	PD / NA	116
8	43 (m)	IFN-Vinbl (PD)	> 1 year	1 (lung)	PD / NA	116+
9	69 (m)	None	< 1 year	2 (lung, LN)	**PR / PR**	116+
10	64 (m)	None	< 1 year	2 (lung, LN)	Early PD / NA	9 §
11	49 (f)	None	< 1 year	2 (lung, LN)	PD / NA	18 §
12	57 (m)	None	< 1 year	1 (LN)	Early PD / NA	13 §
13	52 (m)	None	> 1 year	2 (lung, LN)	PD / NA	141+
14	47 (f)	None	> 1 year	1 (lung)	Early PD / NA	35 §
15	67 (m)	IFN + IL-2 (PD)	< 1 year	2 (lung, LN)	PD / NA	44
16	57 (f)	None	> 1 year	2 (lung, liver)	Early PD / NA	73 §
17	74 (f)	None	< 1 year	1 (lung)	**SD / SD**	115+
18	62 (m)	None	> 1 year	2 (LN, kidney)	Early PD / NA	5 §
19	36 (m)	None	< 1 year	2 (lung, LN)	PD / NA	69
20	70 (m)	None	< 1 year	1 (LN)	PD / NA	24
21	71 (f)	None	> 1 year	4 (lung, LN, liver, kidney)	**SD / SD**	59 §
22	68 (f)	None	> 1 year	2 (lung, LN)	PD / NA	114+
23	62 (m)	IFN + Cis-Retinoid (SD)	> 1 year	1 (lung)	**SD / SD**	89
24	59 (m)	None	> 1 year	2 (lung, kidney)	PD / NA	49 §
26	59 (f)	None	> 1 year	2 (LN)	PD / NA	99+
27	64 (m)	None	< 1 year	2 (lung, LN)	Early PD / NA	90+
28	60 (m)	IFN + Cis-Retinoid (SD)	> 1 year	2 (lung, LN)	**SD / PD**	55 §
29	61 (m)	None	> 1 year	3 (lung, LN, liver)	PD / NA	54+
30	70 (m)	IFN, IL-2, 5FU (SD)	> 1 year	2 (lung, LN)	**SD / SD**	111+
31	75 (m)	None	> 1 year	2 (lung, adrenal)	PD / NA	96
32	60 (m)	None	< 1 year	3 (lung, kidney, adrenal)	**SD / PD**	40+
33	47 (m)	IFN, IL-2, 5FU (SD)	> 1 year	1 (lung)	**SD / PD**	55 §
34	61 (m)	None	> 1 year	3 (lung, LN, adrenal)	PD / NA	105+
35	68 (m)	None	< 1 year	1 (LN)	**SD / PD**	83+
36	61 (f)	IFN (PD)	> 1 year	2 (lung, kidney)	PD / NA	100+

The characteristics of the evaluated patients.
Abbreviations: m=male, f=female, IL-2= Interleukin-2, IFN= Interferon-alpha, 5FU= 5-fluorouracil, Vinbl= Vinblastin, SD= Stable disease, PD= Progressive disease, PR= Partial response, LN= lymphnode, NA= not applicable, + = follow-up ongoing, § = patient deceased.

performed by independent central review.

6.4 Results

Patient characteristics:

Thirty-Six patients with objective progressive mRCC and a Karnofski Performance Status of > 90% were included in this study. Patient 25 was removed from the protocol because after pathology revision the primary tumor was diagnosed as non-clear cell RCC. In 5 patients (12; 14; 16; 18; 27) progression occurred within 6 weeks after study entry and for those patients an additional patient was included, in accordance with the protocol. Characteristics of evaluated patients are given in table 2.

Safety evaluation:

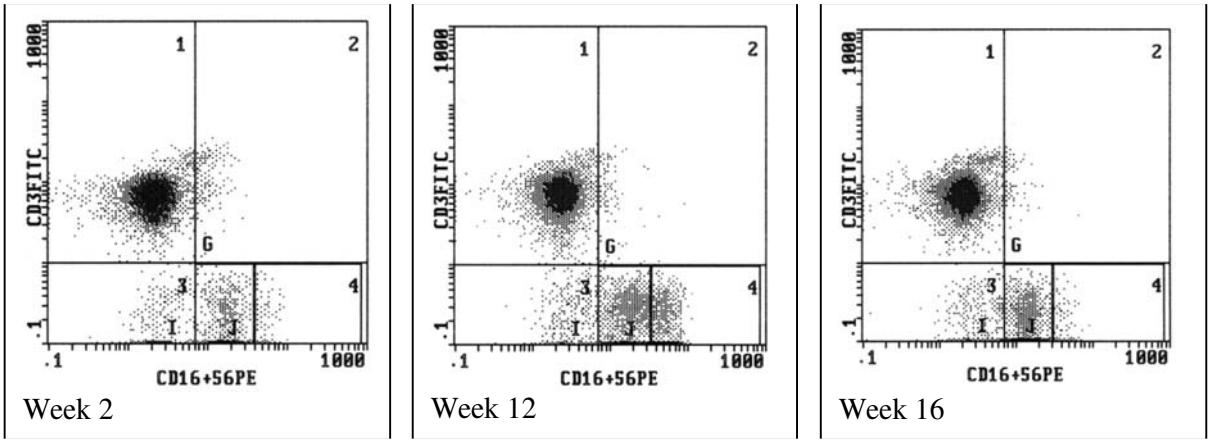
Thirty-five patients received at least 1 infusion of WX-G250, and were assessed for safety. A detailed description of adverse events and severity is shown in table 3. No dose reduction was necessary for WX-G250. During the course of the study, the 35 treated patients experienced a total of 424 adverse events. Patient 18 died within 30 days after the last application of the study medication due to rapid progression of the disease. Four more serious adverse events (SAEs) were observed (pts 10, 12, 14, 16). Three SAE were clearly related to disease progression (pts

Table 3
Adverse events registered during the study, stratified by NCI class and Grade.

NCI class	*Events (N)*	*Pts (N)*	*Pts (%)*	*n.a. (N)*	*Gr I*	*Gr II*	*Gr III*	*Gr IV*
Constitutional Symptoms	225	32	91.4	2	170	50	3	0
Gastrointestinal	88	27	77.1	0	77	11	0	0
Pain	36	18	51.4	0	25	7	4	0
Pulmonary	19	12	34.3	0	12	4	3	0
Dermatology / Skin	12	9	25.7	0	8	4	0	0
Blood/ Bone marrow	10	5	14.3	0	3	3	4	0
Hepatic	9	1	2.9	0	1	5	3	0
Neurology	7	7	20.0	0	6	1	0	0
Lymphatics	4	4	11.4	1	3	0	0	0
Cardiovasculair (General)	4	3	8.6	0	3	1	0	0
Not to classify	2	2	5.7	0	2	0	0	0
Renal/ Genitourinary	2	2	5.7	0	0	1	0	1
Cardiovasculair (Arrhythmia)	1	1	2.9	0	0	1	0	0
Hemorrhage	1	1	2.9	0	1	0	0	0
Infection/ Febrile Neutropenia	1	1	2.9	0	1	0	0	0
Metabolic/ Laboratory	1	1	2.9	0	0	0	0	1
Endocrine	1	1	2.9	0	0	1	0	0
Total	423	35	100.0	3	312	89	17	2

Abbreviations: N=number, %=percentage, Pts= Patients, Gr= Grade, n.a.= Grade not specified.

Figure 1
Representative FACS analysis of peripheral blood mononuclear cell phenotyping



Phenotypic analysis of peripheral blood mononuclear cells by FACS. The CD56+bright population (quadrant 4) was visually estimated. Note the increase of the CD56+bright population during IL-2 treatment and subsequent normalization.

10, 12, 14). The SAE of patient 16 was considered to be probably IL-2 related.

Evaluation of HACA:

HACA levels above the limit of quantification were measured in sera of 2 out of 28 tested patients (7.1%). The maximum HACA level (± 350 ng/ml) of one patient was reached at the end of the 1st course of the treatment (week 12) and remained above limit of detection during follow-up, whereas in the serum of another patient low HACA levels (± 50 ng/ml) only became detectable in week 16 and were again undetectable in week 26. One patient had HACA-levels above limit of detection but below limit of quantification (3.6%) and 25 patients (89.3%) did not develop any detectable HACA at all.

Phenotypic analysis and ADCC of PBMC:

Twenty-eight patients were analyzed. In 50% (14/28) of the patients the absolute blood count number increased more than twofold after treatment initiation. Phenotypic analysis showed that within the total population of leucocytes, the percentage of CD3+ (T-cells) remained constant during the study period, whereas the percentage of CD56+ (Natural Killer (NK)-cells) increased more than a two-fold in 61% (17/28) of the patients. In almost all patients a sharp increase in the CD56bright cell subpopulation was observed and generally CD56bright levels declined to baseline levels after IL-2 discontinuation (fig. 1).

Comparison of LU at baseline, week 10/12 and week 16 for WX-G250-mediated-ADCC and NK-cell-mediated cytotoxicity did not show a significant increase in WX-G250-mediated-ADCC on cell-to-cell basis (fig. 2a). However, since the absolute blood count number increa-

Figure 2
Comparison of Lytic Units measured at baseline, week 10/12 and week 16

Fig. 2a: ADCC, Total Lytic Units
(SKRC-52 cells)

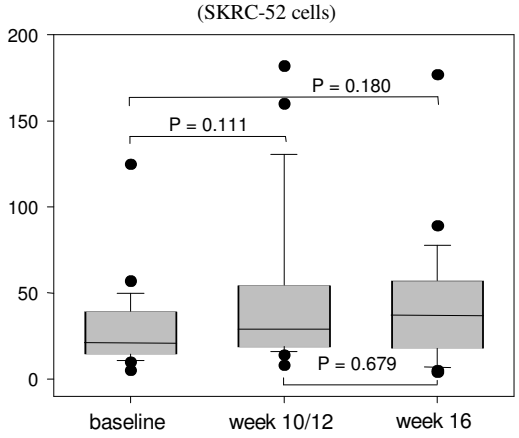
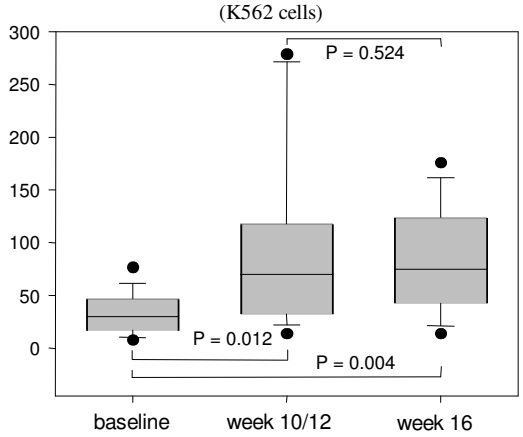


Fig. 2b: Natural Killer Cells, Total Lytic Units
(K562 cells)



Pooled data of the patients in which Lytic Units were measured at baseline, week 10/12 and week 16. The box-plot shows the 25 and 75 percentiles and the standard deviation. The middle of the box represents the mean. The black dots represents the range. ADCC= Antibody Dependent Cellular Cytotoxicity. SKRC-52 is a CA-IXpos cell-line. K562 is an NK-sensitive cell-line.

sed in many patients the total lytic capacity of these patients increased. A significant higher lytic capacity against K562; an NK-sensitive cell-line was observed (baseline/week 16: p=0.004, fig. 2).

No correlation between the percentages of CD3+/16+56+brightcells, the percentages of CD3+/16+56+dim cells, or lytic activity was observed. No relation between disease outcome and ADCC and/or NK activity was observed.

Clinical response and survival:

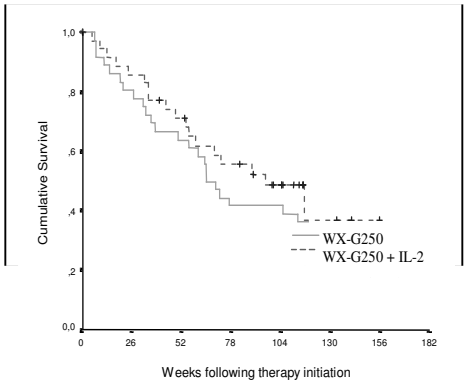
All patients who received at least one dose of WX-G250 (N=35) were included in the efficacy analysis. The median survival was 22 months (fig 3). Clinical progression was seen in 6 patients before week 16. In 5 of these patients (12; 14; 16; 18; 27) progression occurred within 6 weeks after study entry and for those patients an additional patient was included, in accordance with the protocol. One patient (#10) died before week 16, leaving 29 patients for independent radiological evaluation at week 16 revealing 17 PD, 11 SD and 1 PR (Table 2). Subsequently, 12 patients continued treatment. Independent assessment of the clinical status of these 12 patients 4 weeks after the last WX-G250 infusion showed 1x PR and 7x SD. These patients were routinely followed-up by regular CT-scans showing a second partial responder at week 37. Patients receiving prior immunotherapy did not show a different response/survival pattern.

6.5 Discussion

We performed a study with WX-G250 and IL-2 for patients with progressive mRCC of the clear cell subtype. Although the primary trial objective (objective response =15%) was not reached, two partial responses were observed (objective response rate 5.7%) and six additional patients showed stabilization of the disease > 24 weeks. Commonly, SD >24 weeks is recognized as clinical benefit for patients with mRCC with objective progression at entry of treatment. I.e., a clinical benefit was achieved for 23% of our patients. Mean survival was 24 months, which compares favorably with WX-G250 monotherapy, which showed a median survival of 16 months [13], suggestive of an additive/synergistic effect. Clearly, a randomized trial would be necessary to be able to compare both treatments.

Cytokine-based therapies for mRCC lead to a clear survival benefit for responding patients; overall response rate is approximately 15%, with 5% of the patients showing complete responses [2]. Unfortunately, the toxicity profile of these cytokine regimens is significant. The overall survival of the combination treatment we studied in this trial appears to be at least equal to the cytokine-based immunotherapy, but without the unfavorable toxicity profile.

Figure 3:
Overall survival of patients treated with WX-G250 monotherapy and WX-G250 plus IL-2



Kaplan-Meier analysis of overall survival of patients with mRCC treated with WX-G250 monotherapy (N=36) or WX-G250 in combination with a LD-IL-2 schedule (N=35). The analysis of WX-G250 monotherapy treated patients was updated from Bleumer et al. (ref 13). The inclusion of WX-G250 monotherapy patients in this figure does not mean to imply randomization between WX-G250 monotherapy and WX-G250 + LD-IL-2.

The side effects of WX-G250/IL-2 were related to the IL-2 treatment and not to WX-G250: more side effects were encountered during the pulsing days of the LD-IL-2 regimen. WX-G250 monotherapy for patients with mRCC already showed that WX-G250 had a favorable toxicity profile [13].

In 3 patients HACA was detected, albeit that the levels were low (maximum of 350 μg/ml). This low level is in accordance with earlier trials with WX-G250 [10,13], and may be insignificant. Indeed, retreatment of the patient with the highest HACA level did not lead to any adverse effect. WX-G250 appears to be immunological silent in the majority of patients when administered weekly.

We used a LD-IL-2 scheme shown to effect in-vivo expansion and activation of NK-cells [16]. Indeed, the total

blood count of PBMC increased more than a two-fold in 50% of the patients. The relative amount of T-cells (CD3+) did not change during the study period, whereas the percentage of NK-cells (CD3-/56+) did increase in most of the patients. Especially the CD56+bright NK-subset showed an impressive increase. This increase of activated ADCC effector cells was not observed in earlier trials with the unconjugated WX-G250 as monotherapy [13] showing that the IL-2 regimen caused the anticipated effect.

In-vitro studies showed that WX-G250 specific ADCC could be up-regulated with low doses of IL-2 [14,15]. Nevertheless, ADCC expressed as LU per 107 cells, showed no significant increase. Cytotoxic activity against the NK-sensitive cell-line K562 increased significantly between baseline and week 10/12 and week 16. I.e., the potency of the individual effector cells did not increase, but patients experiencing a significant raise in NK cells developed a higher lytic capacity. Although we hypothesized that the effector mechanism of WX-G250 might be ADCC related, no correlation between ADCC levels and the clinical outcome of the study population was observed. The increase in the total lytic capacity together with the observed clinical results still suggests a role for ADCC in tumor cell kill despite our failure to correlate clinical outcome and ADCC. Possibly, the 4-hour 51Cr release assay does not reflect in-vivo RCC cell-kill.

It is possible, but unlikely, that the favorable clinical results were the outcome of the LD-IL-2 regimen. Clinical efficacy for low-dose IL-2 treatment has been described at IL-2 doses that were at least 6-fold higher than the dose of IL-2 used in the current trial [17,18]. Because the previous trial with WX-G250 already showed a clinical benefit, we consider it more likely that the LD-IL-2 regimen and WX-G250 act in a synergic fashion.

Several antiangiogenic therapies are being explored for the treatment of mRCC. Treatment with bevacizumab (anti-VEGF, 10mg/kg/2 weeks)[6] resulted in significantly longer progression-free survival (median: 4.8 months) than in the placebo group (median: 2.5 months), ensuing in closure of accrual on the basis of these differences, despite an overall response rate of approximately 10%, and no difference in survival between the three study-arms. The results of SU11248 as second-line therapy for patients with mRCC were reported recently [4]. SU11248 is an oral multi-targeted tyrosine kinase inhibitor of the PDGF-receptor and the VEGF-receptor. An impressive PR of 40%, time to progression of 8.3 months and median survival of 16 months were observed, and validation of efficacy is commencing. BAY [5], is another oral multi-targeted tyrosine kinase inhibitor, inhibiting Raf kinase and the VEGF-receptor. In a phase III, double-blind trial BAY significantly prolonged progression-free survival compared with placebo in patients with previously treated mRCC. The activity of BAY appears to be similar to WX-G250, whereas SU11248 appears to be more potent. However, becau-

se different endpoints were defined, it is difficult to compare the relative merit of WX-G250, bevacizumab, BAY, and SU11248.

In conclusion, combination therapy of WX-G250 and LD-IL-2 appears to result in a clinical benefit for progressive mRCC patients. Together with a median survival of 22 months this combination is superior to WX-G250 monotherapy and is at least comparable with current non-specific cytokine regimens for patients with advanced mRCC.

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CHAPTER 7

SUMMARY & FUTURE PERSPECTIVES

Summary

Renal cell carcinoma (RCC) is the most prevalent malignancy of the kidney. Due to improved radiological evaluation over 50% of the renal cancers are found incidentally. Despite the fact that these incidentalomas are often confined to the kidney, around 50% of all patients diagnosed with kidney cancer will develop metastatic disease. Metastatic RCC has a poor prognosis. Traditional treatment modalities like chemo- and radiotherapy show overall response percentages of 2-6%. In view of the observed spontaneous remissions of advanced renal cancer, immune mechanisms have been suggested to play a role in the natural disease course of RCC. At present, several non-specific cytokine regimens are used in the treatment of metastatic RCC, e.g. interleukin-2 and interferon-alpha, in combination or as monotherapy or in combination with substances like 13-cis-retinoic acid and/or 5-fluorouracil. Collective data of trials evaluating cytokine-based therapies for metastatic RCC show an overall response rate of approximately 15%, with 5% of the patients showing complete responses. More importantly, cytokine treatment clearly translates into a significant survival benefit in a subset of patients. Nevertheless, the toxicity profile of these cytokine regimens is significant. Therefore, the identification of prognostic factors and biomarkers is of the greatest importance. They may show additional prognostic value over classical prognostic factors like stage and grade. Also, these markers can be used for a better patient selection, development of specific gene-immunotherapy strategies and a better follow-up. In chapter 1 current and future biomarkers of interest in the diagnosis, treatment and follow-up of patients with RCC are reviewed. Furthermore, the different immunotherapeutical modalities currently used are discussed in detail.

Outcome prediction for patients with RCC is based on a combination of factors. In chapter 2 a previously published clinical outcome algorithm based on the 1997 T stage, Fuhrman grade and performance score which stratifies patients into low, intermediate and high risk groups is validated using an international database. A retrospective analysis was performed on 1,060 patients from Nijmegen, the Netherlands; MD Anderson, Houston, USA and the University of California, Los Angeles, USA (UCLA). Patients had localized RCC and were evaluated for outcome prediction using this algorithm. Validation was performed by comparing the 3 risk groups separately within the 3 institutes as well as by comparing hazard ratios and concordance indices among the 3 centers. The estimated disease specific survival rates at 5 years for the low risk groups were 94% (Nijmegen), 92% (MD Anderson) and 93% (UCLA). The 5-year disease specific survival rates for the intermediate risk groups were 65% (Nijmegen), 73% (MD Anderson) and 78% (UCLA), while the rates for the high-risk groups were 40%

(Nijmegen), 30% (MD Anderson) and 48% (UCLA). The concordance indices for each of the databases were 79% (Nijmegen), 86% (MD Anderson) and 84% (UCLA). In conclusion, a clinical algorithm that uses only 3 prognostic variables (1997 T stage, Fuhrman grade and performance status) to stratify patients with localized renal cell carcinoma into 3 risk groups has been shown to be applicable to external databases. This algorithm may be useful for patient counselling, surveillance and identification of high-risk patients for enrolment in clinical trials.

In chapters 3 and 4 the role of dendritic cells (DC) in the development of specific immunotherapy for RCC is discussed. DC have been identified as the most potent antigen presenting cells of the immune system and collective results of DC-based clinical trials show the safety and feasibility of this approach.
In chapter 3, a clinical trial is discussed in which twelve patients with metastatic RCC received a vaccine consisting of autologous immature DC loaded with autologous tumor lysate. The treatment was combined with low-dose interleukin-2, as this has shown benefit in DC-based therapies. Patients received three intradermal vaccinations at two weekly intervals, and, after each vaccination, interleukin-2 was administered for 5 consecutive days. In six patients, Keyhole Limpet Hemocyanine (KLH) was added to the DC culture. KLH is a potent immunogenic neo-antigen that principally stimulates proliferation of T-helper cells. Furthermore, KLH can be used as an immunological control. The vaccine was able to elicit cellular anti-KLH responses, emphasizing the ability of the injected DC to mount an immunologic response. However, proliferative responses against tumor lysate were not detected, and humoral responses against tumor lysate or KLH were absent. Objective clinical responses were not observed, but extended stable disease was noted. The absence of cellular, humoral, or clinical antitumor responses suggests that the vaccination strategy with immature DC has little benefit for patients with advanced RCC. Nevertheless, this study shows the feasibility of a completely autologous DC and tissue culture methodology for the generation of tumor lysate pulsed DC.

In chapter 4, a phase-I clinical trial is described involving CA9-peptide loaded dendritic cells in patients with progressive, cytokine-refractory metastatic renal cell carcinoma. Carbonic Anhydrase-IXG250/MN (CA9) is a renal cell carcinoma (RCC)-associated antigen ubiquitously expressed in the clear-cell subtype of RCC and two CA9-derived peptides have been identified defining a CTL epitope and HLA-DR epitope respectively able to induce T cell responses in vitro. Primary objectives were assessment of the safety, toxicity and induction of CA9-specific immunity.

Six patients with objective progressive metastatic RCC received 5 vaccinations of mature dendritic cells (mDC) pulsed with the CA9-derived peptides and KLH. Peripheral blood was collected at regular intervals, delayed-type hypersensitivity (DTH) was tested at baseline and after the last vaccination and skin biopsies of positive DTH-sites were collected for immunomonitoring purposes. Patients were also monitored for clinical responses. No significant toxicity was observed. In all patients humoral responses were observed against KLH, as well as DTH conversion. Evaluation of biopsy material suggested increased influx of T-helper cells. In none of the immunomonitoring assays evidence for the induction of CA9-peptide specific immunity was observed. No clinical responses were observed. The lack of induction of CA9-peptide specific immune responses indicates that this particular peptide combination cannot induce peptide-specific immune responses.

The concept of selective tumor targeting with antibodies is based on the avid interaction between the antibody and an antigen that is expressed on malignant cells, but not on normal tissues. This specificity can be employed to guide toxic substances or radionuclides to the tumor, but unconjugated antibodies are also able to induce cell death through a variety of mechanisms such as antibody dependent cellular cytotoxicity (ADCC).
In RCC, the antibody WX-G250 has been identified and developed for both diagnostic and therapeutic purposes. It recognizes the CA9 antigen that is expressed in >95% of the clear cell RCC. ADCC is suggested as the main effector mechanism of the ungonjugated WX-250 to induce cell lysis of RCC. Furthermore, in-vitro studies have shown that several cytokines, e.g. interleukin-2 and tumor necrosis factor-alpha, are able two upregulate WX-G250-mediated ADCC. Consequently, two clinical trials were initiated. The results are presented in chapters 5 and 6.

The first trial, discussed in chapter 5, evaluated the toxicity profile, immunogenicity and effectivity of WX-G250 monotherapy. WX-G250 was given weekly by intravenous infusion for 12 weeks. Patients showing stable disease or a clinical response after first treatment cycle were eligible to receive additional treatment for 8 weeks. The weekly schedule of WX-G250 was well tolerated. In all, 10 patients had stable disease and received extended treatment. In one patient a complete response was observed and in another patient significant tumor regression was observed during the follow-up of the treatment. Five patients with progressive disease at study entry were stable for more than 6 months after study entry. With a median survival of 15 months after the start of this treatment and two late clinical responses, WX-G250 seems to be able to modulate metastatic RCC.

Because the number of activated ADCC-effector cells can be increased by low-dose interleukin-2, a multicenter study, discussed in chapter 6, was initiated to investigate whether WX-G250 combined with low-dose interleukin-2 can lead to improved clinical outcome of patients with progressive metastatic RCC. Thirty-five progressive RCC patients received weekly infusions of WX-G250 for 11 weeks combined with a daily low-dose interleukin-2 regimen. Patients were monitored longitudinally for ADCC capacity. Radiological assessment of the metastatic lesions was performed at week 16 and regularly until disease progression. A durable clinical benefit was achieved in 8 of 35 patients (23%; 3x partial response, 5x stabilization >24 weeks). In general the treatment was well tolerated with little toxicity. In conclusion, combination therapy of the chimeric monoclonal antibody WX-G250 and low-dose IL-2 pulsing appears to result in a clinically significant benefit for progressive metastatic RCC patients. Together with a median survival of 22 months the combination of WX-G250 and low dose IL-2 is superior to WX-G250 monotherapy and seems comparable with current non-specific cytokine regimens for patients with advanced RCC.

Future perspectives

As discussed in this thesis, a clinical algorithm using 3 well-known prognostic factors (1997 T stage, Fuhrman grade and performance status) can be used to stratify patients that undergo nephrectomy for localized disease into 3 risk groups. This is useful for patient counselling and useful to choose the optimal post-operative management for the individual patient. The results indicate that watchful waiting is adequate treatment for patients in the low risk group only, while patients in the intermediate risk group, but surely patients in the high risk might benefit from adjuvant treatment. Unfortunately, little is known about adequate adjuvant therapy for patients with a high risk for recurrent disease. Given the results with WX-G250 in metastatic RCC, it will be of interest to evaluate the effect of WX-G250 treatment in an adjuvant setting.

The ideal staging system would stratify patients into 2 risk groups with a 0 and 100% probability of recurrent disease respectively. Unfortunately, with the current clinical and pathological parameters this is not possible. Particularly, the majority of patients fall in the intermediate risk group. Hopefully, the increasing knowledge of molecular markers and gene profiling will increase the power to discriminate between RCC risk groups, as has been shown for e.g. breast carcinoma patients. Several markers discussed in chapter 1, e.g. CA9, are suggested to give independent prognostic information, however, more research is needed to obtain new (better) antigens and markers in RCC. Expression array analyses have shown promise, but large cohorts need to be studied before prognostic gene expression signatures become available.

The two clinical trials that are discussed in chapters 3 and 4 clearly show the difficulties of current dendritic cell-based cancer vaccine research. The feasibility and safety of the approach is clear but the efficacy is unsatisfactory and to what extend immune responses are directed specifically against the tumor remains unknown. It is frustrating that dendritic cells, regardless the phenotype, are able to induce tumor-specific immune responses in-vitro and in animal studies but fail to do so in the majority of vaccinated patients. Of course, most tumor-associated antigens used in cancer vaccines (including CA9) are essentially self-antigens, with patients being in a tolorogenic state. This might explain why the CA9-derived peptides used in our study did not show induction of CTL or humoral responses, despite in-vitro results that show activation of both CD4+ and CD8+ T-cells directed against the peptides. Nevertheless, parallel studies in melanoma have been successful, at least in the demonstration of induction of peptide-specific CTL. The more intriguing are the results of another CA9 peptide vaccination trial with HLA-

A24 restricted peptides, in which CA9 specific, and more importantly, clinical responses were observed. Noteworthy is that the responses were observed after numerous vaccines (>11 biweekly vaccinations). Perhaps those peptides are simply more immunogenic. Alternatively, the extended vaccination might imply that long-lasting immunotherapy is needed to break local tolerance and/or the immunosuppressive state.
This is consistent with recent insight in tumor immunology revealing that local immunosuppression might be one of the major obstacles in the development of an effective immunotherapy strategy. On top of immunosuppression, tumor escape through e.g. downregulation of HLA molecules or Fas regulation may alter the outcome of immunotherapy. Thus, counterbalancing immunosuppression with at the same time potent vaccine strategies might eventually lead to effective immunotherapies. Indeed, vaccination combined with measures to deplete regulatory T cells (ONTAK) or blocking T cell downregulation by blocking CTLA-4 has shown robust anti-tumor responses. However, CTLA4 is important in maintaining immune homeostasis, and anti-CTLA4 treatment does provoke autoimmune toxicity. Thus far, ONTAK treatment has not resulted in autoimmune toxicity. Thus a delicate balance between tumor immunity and autoimmunity exists. A key issue of future research is to determine whether tumor destruction and severe autoimmunity can be dissociated, e.g. through manipulation of (antigen) specific Treg populations.

The two clinical studies presented in chapters 5 and 6 indicate that the monoclonal antibody WX-G250 might play a role in the treatment of RCC. The clinical results seem at least comparable with the high-dose non-specific cytokine regimens but with a very favourable toxicity profile. Consequently, phase-III trials are needed to compare the efficacy of WX-G250 with the efficacy of the current non-specific cytokine treatments for patients with metastatic RCC. As mentioned, WX-G250 may be useful in adjuvant treatment for patients macroscopically free of RCC after nephrectomy with intermediate/high risk for recurrent disease. The clinical studies failed to confirm ADCC as WX-G250 effector mechanism, although in vitro studies clearly demonstrated the reverse. Therefore, fundamental research remains essential to gather more insight into the working mechanism of WX-G250.

So far, the development of specific immunotherapy for cancer has focussed either on the induction of cellular immune responses through the use of vaccines or passive immunotherapy by infusion of monoclonal antibodies. However, combining these strategies may induce a more powerful anti-tumor effect. DC pulsed with tumor cells coated with monoclonal antibodies seem to be able to present the tumor antigens with superior efficiency DC loaded with

irradiated tumor cells or tumor specific peptides alone. Given the high presence of the CA9 antigen on RCC cells, the WX-G250 antibody will be ideal to explore this strategy in the RCC setting.

As discussed in chapter 1, mutations in the VHL gene are found in over 75 % of clear cell RCC. Many of the gene products that are upregulated as a consequence of these mutations are involved in angiogenesis and proliferation. Subsequently, new therapies are being explored to block these growth factors. These new treatment modalities include anti-VEGF monoclonal antibodies (bevacizumab), tyrosine kinase inhibitors (SU-11248), targeting of the Raf kinase pathway (BAY 43-9006) and inhibition of the mTOR pathway (CCI-779). Based on the first promising results, further exploration of these compounds has to establish their place in the treatment of RCC. In particular, it will be interesting to combine various treatments: e.g. VEGF-induced immunosuppression can be inhibited by bevacizumab, which might be beneficial in vaccination strategies. Secondly, the extend of clinical cross-resistance is unknown, which provides a rational for combination. Extensive, complicated clinical trials are currently being designed to compare various combination strategies, all aimed at defining a safe and effective treatment for metastatic RCC patients.

CHAPTER 8

SAMENVATTING

Hoofdstuk 1

Het niercelcarcinoom is de meest voorkomende kanker van de nier, waarvan de incidentie aan het toenemen is. Door verbeterde radiologische technieken wordt tegenwoordig tot meer dan 50% van alle niercelcarcinomen bij toeval ontdekt. Ondanks het gegeven dat deze zogenoemde incidentaloma's zich over het algemeen binnen de nier begeven, ontwikkelt de helft van alle patiënten gediagnosticeerd met niercelcarcinoom systemische ziekte. Uitgezaaid niercelcarcinoom heeft een zeer slechte prognose. Traditionele behandelingen zoals chemo- en/of radiotherapie laten een respons percentage zien van 2-6%.
Aangezien spontane remissies van uitgezaaid niercelcarcinoom in de literatuur worden beschreven, wordt aangenomen dat het afweersysteem een rol speelt in het ziektebeloop van niercelcarcinoom. Dit heeft geleid tot de ontwikkeling van non-specifieke immunotherapie en tegenwoordig kan de behandeling van uitgezaaid niercelcarcinoom door middel van een op cytokinen gebaseerde therapie als standaard beschouwd worden. Deze therapieën bestaan uit de cytokinen interleukine-2, interferon-alfa, een combinatie van deze twee of zelfs nog uitgebreid met 5-fluorouracil. Wanneer men de uitkomsten van verscheidene studies naar deze cytokinen in de behandeling van het uitgezaaide niercelcarcinoom met elkaar vergelijkt, blijkt dat bij ongeveer 15% van de patiënten een respons te zien is, en bij 5% zelfs een complete remissie van de ziekte. Belangrijker nog, behandeling met cytokinen leidt tot een winst in de overleving. Desalniettemin is het bijwerkingen-profiel van deze non-specifieke cytokinen-therapieën significant. Vandaar dat de zoektocht naar prognostische factoren en niercelcarcinoom-specifieke eiwitten zo van belang is. Deze kunnen een toegevoegde waarde hebben in een meer adequate patiëntenselectie voor eventuele verdere behandeling. Daarbij kunnen deze eiwitten de basis zijn voor de ontwikkeling van meer specifieke immuno-gen therapieën. In hoofdstuk 1 wordt een introductie gegeven van het niercelcarcinoom, het immuun systeem en worden de verschillende immunotherapieën die op dit moment in gebruik zijn bediscussieerd. Tevens worden verschillende eiwitten beschreven die van belang (kunnen) zijn in diagnostiek, behandeling en follow-up van patiënten met niercelcarcinoom.

Hoofdstuk 2

Met de huidige ontwikkeling van beeldvormende technieken wordt het niercelcarcinoom steeds eerder ontdekt. Samen met de ontwikkeling van de immunotherapie voor het gevorderde niercelcarcinoom heeft dit geleid tot een verbeterde prognose voor niercelcarcinoom.

Hierdoor is een vernieuwing nodig van de bestaande prognostische stadiëring classificaties. Bovendien kunnen in de toekomst op deze wijze subgroepen van patiënten worden geïdentificeerd die beter zullen reageren op al dan niet specifieke immunotherapie. Vandaar dat het UCLA de geïntegreerde stadiëring classificatie, UISS heeft ontwikkeld. De UISS heeft de TNM-classificatie uit 1997 geïntegreerd met de Fuhrman pathologische gradering en de ECOG performance status. Op deze wijze is het mogelijk op meer individuele wijze patiënten te karakteriseren.
Het doel van de studie beschreven in hoofdstuk 2 was de validatie van de UISS aan de hand van een database van patiënten met niet uitgezaaid niercelcarcinoom behandeld in respectievelijk het UCLA (Los Angeles), MD-Anderson (Houston) en het UMC St. Radboud te Nijmegen. In totaal zijn de gegevens van 1.060 patiënten gebruikt voor de validatie. De validatie is uitgevoerd door de patiënten onder te verdelen volgens de UISS om vervolgens de specifieke overleving van de 3 prognostische groepen te vergelijken tussen en binnen de verschillende centra. De volgende 5-jaars overlevingen werden berekend naar aanleiding van de onderverdeling: de laag-risico groep 94% (Nijmegen), 92% (Anderson) en 93% (UCLA); de gemiddelde-risico groep 65% (Nijmegen), 73% (Anderson) en 78% (UCLA); de hoog-risico groep 40% Nijmegen, 30% (Anderson) en 48% UCLA.
Deze studie laat zien dat een klinisch algoritme van slechts 3 prognostische factoren in staat is om patiënten met niet uitgezaaid niercelcarcinoom adequaat onder te verdelen in 3 risicogroepen. Met behulp van deze risico groepen kan vervolgens een toepasselijke begeleiding en behandeling worden uitgezocht op de individuele patiënt.

Hoofdstuk 3 & 4

In de behandeling van het uitgezaaide niercelcarcinoom wordt de aandacht steeds meer verlegd van non-specifieke immunotherapie, zoals interleukine-2 of interferon-alpha, naar meer specifieke immuuntherapieën. Hierbij wordt onderandere gebruik gemaakt van dendritische cel vaccinaties. Dendritische cellen (DC) zijn de meest potente antigeen presenterende cellen van het menselijk lichaam die in-vitro gekweekt kunnen worden en dus zeer geschikt zijn voor de inductie van een tumor specifieke immuunrespons.
In hoofdstuk 3 wordt een studie beschreven waarbij 12 patiënten met uitgezaaid niercelcarcinoom na de verwijdering van de nier een vaccinatie hebben gehad met DC geladen met tumorcellen. De vaccinaties werden gecombineerd met een interleukine-2 behandeling. De drie vaccinaties werden om de week in de huid gegeven. Voor, tijdens en na de uitvoering van de stu-

die is bloed afgenomen voor immunologische monitoring. De uitzaaiingen zijn door middel van CT-scans geëvalueerd.
Deze studie laat de veiligheid en haalbaarheid zien van vaccinaties op basis van DC beladen met tumorcellen. Immunologische monitoring liet een milde reactie zien op KLH, een sterk immunogeen en lichaamsvreemd controle antigeen. Tumorspecifieke immuunreacties zijn niet geobserveerd. Geen van de patiënten toonde afname van de uitzaaiingen bij klinische evaluatie, al zijn er enkelen langdurig stabiel gebleven en uiteindelijk na operatieve verwijdering van de uitzaaiingen vrij van meetbare ziekte. Behalve een voorbijgaande temperatuursverhoging zijn geen bijwerkingen geobserveerd.

Uit diverse studies is gebleken dat er twee populaties DC zijn. Immature DC en mature DC. In deze studie zijn immature DC gebruikt, vanwege optimale opname van antigenen. Mature DC zijn daarentegen superieur in de migratie en de presentatie van de antigenen aan T-cellen en uit verschillende onderzoeken is dan ook gebleken dat het gebruik van mature DC een betere immunologische en klinische respons laat zien.

"Carbonic anhydrase-IX$^{MN/G250}$" (CA9) is een antigeen wat sterk geassocieerd is met het niercelcarcinoom aangezien het in meer dan 95% van het clear-cell subtypen van de niercelcarcinomen tot expressie komt. Onlangs zijn twee peptiden geidentificeerd afkomstig van het CA9 antigeen die herkend worden door cytotoxische T-cellen (type-I peptide) en T-helper cellen (type-II peptide). Dit heeft geleid tot de initatie van de studie zoals beschreven in hoofdstuk 4. In dit klinisch onderzoek hebben zes patiënten met progressief uitgezaaid niercelcarcinoom vaccinaties gekregen van mature DC beladen met de CA9-afgeleide peptdiden. Van deze patiënten is het bloed geevalueerd voor immunologische responsen, uiteraard is ook gekeken naar het klinisch beloop.
Zoals bekend uit onze vorige ervaringen met DC-vacinaties en de literatuur zijn geen noemenswaardige bijwerkingen gezien tijdens de studie. Immunologische monitoring liet duidelijk een sterkere reactie zien tegen KLH in vergelijking tot de vaccinaties met immature DC. De DTH-huidtest ("delayed type hypersensitivity") werd bij alle patiënten positief gedurende de studie voor de DC beladen met zowel KLH als het type-I peptide. Bij de evaluatie van de huidbiopten van deze positieve huidtesten zijn met name T-cellen en cytokinen geobjectiveerd die betrokken zijn bij het T-helper "pathway", wat impliceert dat de immunologische reacties gericht zijn tegen KLH.
Bij geen van de patiënten heeft de behandeling geresulteerd in een klinisch effect.
Kortom, de gebruikte vaccinaties zijn in staat om het immuunsysteem te activeren, maar zon-

der dat er een specifieke afweerreactie tegen de gebruikte peptiden is waargenomen. Blijkbaar zijn de peptiden gebruikt is deze studie niet in staat om afweerreacties op te wekken. In de te volgen studies zullen dan ook of het hele antigeen, met alle epitopen, of zelfs hele tumorcellen gebruikt gaan worden om te beladen op de mature DC.

Hoofdstuk 5 & 6

Het gebruik van antilichamen binnen de therapie van kanker is gebaseerd op de sterke binding van het antilichaam met een antigeen wat aanwezig is op de tumor cellen, maar niet op normaal weefsel, een zo genoemd tumor-associated antigen. Deze specificiteit kan gebruikt worden om antilichamen gebonden aan toxische of radioactieve agentia specifiek te sturen naar de tumor dus alleen daar celdood te induceren.
Ook het ongebonden antilichaam kan echter celdood induceren, onder andere door middel van het antibody dependent cellular cytotoxicity, oftewel ADCC. ADCC is gebaseerd op de specifieke binding van een antilichaam met een antigeen. Vervolgens herkennen natural killer cells, NK-cellen, dit antilichaam tumor complex wat leidt tot lyse van de cel.
Voor het niercelcarcinoom is het CA9 eiwit geïdentificeerd. Dit antigeen is aanwezig in meer dan 85% van alle niercelcarcinomen en in >95% niercelcarcinomen van het clearcell subtype. CA9 komt niet tot expressie in normaal nierweefsel.
Het antilichaam G250 bindt aan het CA9 antigeen en is ontwikkeld zowel voor diagnostische als voor therapeutische doeleinden. Het G250 gebonden aan radioactief jodium heeft duidelijk laten zien dat het specifiek target naar de metastases van de niercelcarcinoom. Voor het ongebonden G250 wordt ADCC gezien als belangrijkste mechanisme om celdoor te induceren. Verder is uit in-vitro onderzoek gebleken dat diverse cytokinen, waaronder interleukine-2, in staat zijn G250 gemedieerde ADCC op te reguleren.
Deze ontwikkelingen hebben geleid tot de initiatie van twee klinische studies met G250 voor patiënten met een gevorderd stadium van niercelcarcinoom. Deze beide studies staan beschreven in de hoofdstukken 5 en 6. De eerste studie onderzocht G250 als monotherapie, vervolgens is een studie uitgevoerd die de combinatie onderzocht van G250 met een lage dosis interleukine-2. Doelen van beide studies waren de evaluatie van eventuele bijwerkingen van G250 met of zonder IL-2, en de effectiviteit in de vorm van meetbare ADCC in het perifere bloed en klinische responsen. Patiënten in de combinatie-studie kregen eerst een week IL-2, om IL-2 specifieke bijwerkingen te kunnen monitoren. Vervolgens kregen patiënten een wekelijks infuus van 20mg G250 gecombineerd met dagelijks een lage dosis IL-2 door middel van zelf-

injectie. Om de week werd dit IL-2 schema afgewisseld met drie dagen een hogere dosering IL-2.

In totaal hebben 71 patiënten minstens 1x een infuus gehad met het antilichaam G250. 35 patiënten hiervan in combinatie met een dagelijkse subcutane injectie van interleukine-2. Beide studies laten een zeer gunstig bijwerkingen profiel zien. De meeste bijwerkingen die door de patiënten genoemd zijn, waren milde en voorbijgaande klachten van rillingen, koorts en moeheid. Deze klachten zijn bekend van interleukine-2 gebruik. De dagboeken die de patiënten bijgehouden hebben laten zien dat er duidelijk meer klachten genoteerd zijn tijdens en vlak na de drie dagen hogere dosis IL-2.

Follow-up van de patiënten liet 1 complete response, 4 partiële responders en 11 patiënten zien met stabilisatie van de ziekte van langer dan 24 weken. In het geval van gemetastaseerd niercelcarcinoom wordt dit gezien als een "clinical benefit". Tevens is er een mediale overleving van rond de 17 maanden. Van de combinatie studie is deze zelfs 22 maanden.

Evaluatie van de ADCC-assays die uit het perifere bloed van deze patiënten gedaan zijn laat zien dat de interleukine-2 behandeling een duidelijk effect heeft op de hoeveelheid effector cellen, dus de NK-cellen, in het perifere bloed. De non-specifieke cytotoxiciteit door middel van NK-cellen gedurende de studie is dan ook significant is toegenomen. Specifieke G250-gemedieerde cytotoxiciteit wordt echter helaas niet gezien.

Samenvattend laten deze twee studies ons zien dat G250 geen noemenswaardige bijwerkingen laat zien, en dat de lage dosis interleukine-2 goed te verdragen is. Ondanks het feit dat verschillende in-vitro studies laten zien dat ADCC in staat is niercelcarcinoomcellen te doden, wordt in deze studies geen significante G250-gemedieerde ADCC gezien. Of de rol van ADCC in de in-vivo situatie minder van belang is dan gedacht of dat dit het gevolg is van een lokale immunosuppressie door de tumor cellen moet het onderwerp worden van verdere studies. Desalniettemin wordt bij 22.5% van de patiënten een klinisch voordeel gezien door deze behandeling. Verder is de mediane overleving van 22 maanden die gezien is bij de combinatie studie op zijn minst vergelijkbaar met de op dit moment gebruikte non-specifieke immunotherapieen zoals interferon-alpha en interleukine-2, maar dan zonder de zware bijwerkingen. G250 lijkt dus in staat het beloop van gemetastaseerd niercelcarcinoom positief te beïnvloeden.

CHAPTER 9

DANKWOORD & CURRICULUM VITAE

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Om een co-promotor te hebben die als clinicus zo enthousiast bezig is met de integratie van fundamenteel onderzoek en de (academische) kliniek van alle dag werkt zeer inspirerend. Ik kijk dan ook uit naar het vervolg van onze samenwerking in het verdere traject van mijn opleiding tot uroloog waarbij de kliniek op de voorgrond zal staan.

Geachte dr. E. Oosterwijk, Beste Egbert,
Dankzij de vele brainstormsessies om onze gegevens zo goed mogelijk op papier te zetten is de immunologie verworden van een abstract begrip tot…nou ja, een iets minder abstract begrip. De talloze momenten tussendoor waarin met indrukwekkende geestdrift het werkingsmechanisme van een fagocyterende interleukine secreterende cytotoxische cel werd uitgelegd, compenseert volledig al die manuscripten die rood gekladderd terug in mijn u-tje terecht kwamen.

Beste Dorien en Jeannette,
De lijn tussen promovendus en analist is bij de Experimentele Urologie flinterdun. Samen zijn we bezig geweest met het uitdenken van experimenten en de interpretatie van de verkregen data. Dit gold echter ook de uitvoering van de experimenten. Zeker voor mij als clinicus is dat erg belangrijk geweest door het inzicht die het daadwerkelijk uitvoeren van de experimenten verschaft in de data. Ik kan de lezers echter verzekeren dat ik daar bij het uitvullen van well nummer 649 met 7.5 microliter van het een of het ander daar ook wel eens andere gedachten over heb gehad. Daar staat tegenover dat de gezelligheid op de kweek of het inzetten van de DC-kweek van de patiënten op de clean-room tot 's avonds laat onvergetelijke herinneringen opgeleverd heeft.

Beste prof. dr. J.A. Schalken, Beste Jack & alle medewerkers van experimentele urologie.
Wat hierboven al genoemd is, is uiteraard te extrapoleren naar het hele lab experimentele urologie onder de bezielende leiding van prof. dr. Jack Schalken. De combinatie van levendige

discussies tijdens de WIP- & literatuur besprekingen, en de evenzo levendige gesprekken tijdens de pauze en weekevaluaties bij St Anneke, maken dat de drie jaar op het lab op zeer productieve & ontspannen wijze voorbij zijn gevlogen.

Polikliniek Urologie UMC St Radboud,
De beschreven klinische trials in dit boekje staan of vallen mede met goede opvang van patiënten, begeleiding en uitleg over de studies. Op de polikliniek bleek dit een vanzelfsprekendheid. Vooral de goede en plezierige samenwerking met Anita, José en Suzanne mag dan ook hier niet ontbreken.

Collega's in Apeldoorn:
Na 3 jaar wetenschap was het wel even omschakelen naar de chirurgische kliniek. Gelukkig waren daar de assistenten chirurgie te Apeldoorn: Hard werken, veel lol en vooral soepel ruilen als er tijd nodig was om artikelen in te dienen.

Alle vrienden, vriendinnen, familie en kennissen.
Namen noemen lijkt me niet nodig, iedereen bedankt.

Lieve Fred, Berna, Anoek, Richard, Joeri, Desiree,
Misschien dat jullie na het lezen van de Nederlandse samenvatting enigszins snappen wat ik überhaupt gedaan heb… Desondanks bleven jullie oprecht geïnteresseerd in het verloop van het project en wanneer het feest nou een eindelijk zou volgen.

Lieve Leonie,
Als ik dit dankwoord teruglees, lijkt het wel alsof een promotie project een brok gezelligheid is. Jij weet wel beter! Al die keren dat er weer een cruciale DC-kweek de mist in ging, een manuscript niet geaccepteerd werd, en alle andere denkbare teleurstellingen horend bij een promotieproject, stond jij daar in avonduren en weekeinden klaar om het gezeur te nuanceren, relativeren en zelfs gewoon aan te horen. Maar ook was je daar om me van de bank af te schoppen om door te gaan als het nodig was.
Baie danki, Dushi!

CURRICULUM VITAE

Ivar Bleumer werd geboren op 26 december te Tilburg, maar verhuisde binnen enkele maanden al naar Nijmegen. Na een tropische tussenstop te Curaçao alwaar de eerste drie jaar van het atheneum werden gevolgd aan het Peter Stuyvesant College, vervolgde Ivar zijn opleiding aan het Canisius College Mater Dei te Nijmegen.
In 1993 werd gestart met de opleiding geneeskunde aan de Katholieke Universiteit Nijmegen. Aansluitend aan het halen van de artsenbul in augustus 2000 startte Ivar met het onderzoeksproject "Optimalisatie van specifieke immunotherapie voor het niercelcarcinoom" aan de afdeling Urologie van het Universitair Medisch Centrum St Radboud te Nijmegen. Het resultaat daarvan heeft u in handen. In de 2004 startte Ivar met de opleiding tot uroloog aan de afdeling Heelkunde van Gelre Ziekenhuizen te Apeldoorn (opleider dr. W.H. Bouma) in het kader van de vooropleiding. Momenteel is Ivar werkzaam aan de afdeling Urologie van het Canisius Wilhelmina Ziekenhuis (opleider dr. H.F.M. Karthaus). De opleiding zal worden voltooid aan het Universitair Medisch Centrum St. Radboud te Nijmegen (opleider prof. dr. F.M.J. Debruyne).